Flowering and seed production in *Eucalyptus nitens*

by

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Declarations

This thesis does not contain any material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgment is made in the text of the thesis.

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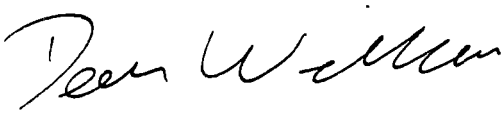

Dean R. Williams

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Abstract

This project examined silvicultural and environment factors which affect precocious and abundant flowering in trees of the economically important plantation species *Eucalyptus nitens* with the aim of optimising seed production.

Two separate studies were undertaken to examine how flowering and seed quality were affected by the macroenvironment. The first studied an altitudinal gradient which would span the range where operational seed orchards might be located. Flowering abundance and seed production was greatest on sites where growth rate was highest. Seed weight, germination success and germination rate decreased as site altitude increased. The second study examined the effects of water availability. Flowering abundance was highest in trees experiencing water stress, whilst seed quality remained unaffected by parental water status. Overall, the maternal tree had a greater influence on seed quality traits than the environmental effects studied.

On the microenvironmental scale, the effect of tree spacing on flower abundance and capsule survival was studied at two sites where trees were 5 and 13 years old. As the spacing between trees increased so too did reproductive yield, not only per tree but also per hectare. Furthermore, it appears that as trees mature, tree density needs to be decreased to maintain the maximum reproductive yield per hectare.

To overcome the strong genetic control of flowering precocity a hormone manipulation approach was tested. The gibberellin biosynthesis inhibitors paclobutrazol, chlormequat chloride and prohexadione were applied to seedlings and their relative effects compared. These treatments reduced both growth rate and endogenous levels of GA_1 to varying degrees, with paclobutrazol the most effective. However, none of the treatments promoted precocious flowering. Further environmental and/or chemical manipulation would be required to induce precocious flowering in *E. nitens* seedlings.

The application of nitrogen fertiliser to juvenile trees stimulated precocious and abundant flowering. This was due in part to accelerated growth rate but nitrogen also acted independently of growth rate. In contrast, phosphorus had no effect on growth or reproductive output but did cause trees to undertake vegetative phase change earlier. Nitrogen fertiliser combined with hormone manipulation with paclobutrazol was applied to juvenile and mature plantation grown trees to promote precocious and abundant flowering. There was an additive effect in combining the treatments in promoting both precocious and abundant flowering.

The production of pedigree seed traditionally required three visits to the mother tree to carry out controlled pollination. To improve the efficiency of this process, a number of novel controlled pollination procedures were tested on both *E. nitens* and *E. globulus*. A new single visit pollination protocol for *E. globulus* was successfully developed, whilst the techniques applied to *E. nitens* yielded no advantage over the traditional method. This new protocol for *E. globulus* is expected to reduce the cost of pedigreed seed production by more than half.

This thesis identifies a number of beneficial practices which will improve the productivity and economic performance of *E. nitens* seed orchards.

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The format of presented chapters

Two of the chapters of this thesis have been published as scientific papers in refereed journals. The structure of these papers has been retained within the thesis with minor changes to maintain the flow and coherence with the accompanying chapters. These changes include:

- the removal of abstracts, summaries and general introductory statements,
- the general acknowledgments and bibliographies have been amalgamated with those of the unpublished chapters of the thesis,
- the addition of chapter numbers to tables and figures.

Each chapter represents a discrete investigation and can stand alone as a report of scientific research.

The refereed publications from this thesis are:

Williams, D.R., Ross, J.J., Reid, J.B. and Potts, B.M. (1999), Response of *Eucalyptus nitens* seedlings to gibberellin biosynthesis inhibitors. *Plant Growth Regulation* **27**: 125-129.

Williams, D.R., Potts, B.M. and Black P.G. (1999). Testing single visit pollination procedures for *Eucalyptus globulus* and *E. nitens*. *Australian Forestry* **61**: 60-66.

This second listed article actually published in volume 62, 1999 on pages 346-352 of Australian forestry

Chapter 1

Introduction

The forest plantation estate in Australia is poised to treble by the year 2020 through planting an average of 80,000 hectares per year (2020 Vision; Anon 1999). The 2020 Vision for Australian plantation forestry aims to reverse the current $1.5 billion trade deficit for forest products and create up to 40,000 jobs in rural areas. This dramatic increase in the plantation estate is driven by economic considerations and political pressure to move production out of native forests and the need to counter greenhouse gas production (Borough *et al.* 1998).

Eucalypt plantations will be a major means of achieving the 2020 Vision target due to the high demand for eucalypt pulp for paper production. The profitability of these plantations will to a large extent depend upon the quality of genetic stock and the initial costs of plantation establishment. The cost of the planting stock is incurred at the beginning of the plantation and must be discounted over a 20 year rotation. Clonal propagation of temperate eucalypts is uneconomic under Australian cost structures and seed is the only alternative for large-scale commercial deployment (Borralho 1997). This has resulted in industry investing heavily in seed orchards. By 2002 most plantation seed is expected to be derived from seed orchards (Tibbits 1997). Investment in seed orchards has occurred by major forestry companies, and medium and small sized enterprises, including farmers, wishing to diversify their production. These investments have been fuelled by the national and international shortage of eucalypt seed and the high demand for elite seed that is

always in short supply. High demand and continued selection of superior genotypes means seed orchards play a critical role in the future productivity of the forest industry.

Eucalyptus globulus and *E. nitens* are the main temperate hardwood plantation eucalypts in Australia (Tibbits 1997) and *E. nitens* is particularly suitable for plantations in frost-prone regions. In Australia, the total plantation area of *E. nitens* in 1995 was approximately 46,000 hectares and this is projected to increase to about 66,000 hectares (or by about 45%) by 2000 (Tibbits 1997). *Eucalyptus nitens* appears to be the more problematic of the two temperate eucalypts with respect to flowering and seed production and is the focus of this thesis.

Flowering in *E. nitens* is subject to the vagaries of the environment in which it grows. In natural stands good flower crops occur infrequently, as little as once every 4 years (Moncur 1998). When trees are taken out of their natural range for domestication it can reduce this frequency further or inhibit flowering altogether as experience has shown in orchard plantings of *E. nitens* in South Africa (Eldridge and Griffin 1990). Where to locate an orchard is one of the most critical and irreversible decisions to be made and necessitates sound knowledge of what are desirable environmental characteristics.

Drier environments have induced heavy flower crops in *E. viminalis* (Moncur 1998) and *E. diversicolor* (Eldridge *et al.* 1993) but appear to substantially reduce flowering in *E. regnans* (Ashton 1975), *E. macrorhyncha* (Ashton and Sandiford 1988) and *E. maculata* (Pook *et al.* 1997). To initiate flowering, *E. nitens* has an obligate requirement for an extended period of cold temperatures (Moncur and Hasan 1994). However, cooler environments delay the onset of flowering and seed maturity, and reduces the production of viable seeds (Moncur *et al.* 1994a). These at first appear to be intractable problems. However, through surveying a range of sites where *E. nitens* is planted and assessing

reproductive performance from flower initiation through to seed production and germination success, a picture may be obtained of what benefits or drawbacks typical environmental characteristics have on reproduction. Chapter 2 reports on such a survey where five sites were monitored and sampled over two consecutive years. Four of these sites were located in an altitudinal transect in south-eastern Tasmania where site elevation ranged from 60 m to 650 m above sea level. The fifth site was an established irrigation trial situated in a low rainfall area of south-eastern Tasmania where water availability to the trees was strictly controlled and monitored.

Once a suitable site has been selected with all the desirable environmental characteristics for seed production one of the next important decisions is the orchard design and more specifically the spacing between trees. A good eucalypt orchard design is one which is the best compromise between: productivity per tree which increases with wider spacing (Moncur 1998), rates of outcrossing which decrease with wider spacing (Potts and Wiltshire 1997) and resource utilisation which becomes more efficient over a smaller area (Bouvet 1997). Despite tree spacing appearing to have important implications on whole eucalypt orchard productivity, it has been given very little attention compared to the improvement in productivity of single trees. Chapter 3 reports on a study of the effect of tree spacing on the production of flowers and capsules by *E. nitens*. This study utilised two established spacing trials and presents the results on both a per tree basis and per hectare basis as alternate measures of assessing productivity.

When the *E. nitens* orchard is established, both in the best location and with the optimal tree spacing for seed production, the time interval until the first good crop may take from 4 to 10 years without further intervention (Moncur 1994). In the closely related *E. globulus*, flower initiation has been chemically induced through application of the gibberellin biosynthesis inhibitor paclobutrazol in trees of less than 2 years of age (Hasan

and Reid 1995). Paclobutrazol is also effective in stimulating flowering in adult trees of *E. nitens* (Griffin *et al.* 1993). However, application of paclobutrazol to juvenile *E. nitens* has had no (Griffin *et al.* 1993) or low (Moncur 1998) rates of success. Furthermore, there are some concerns surrounding the use of paclobutrazol regarding its high levels of residual activity, the persistence of which has been detected for up to 6 years after treatment (Hetherington and Jones 1990). There is a strong relationship between reduced levels of apical GA_1 and increased flowering abundance in *E. nitens* (Moncur and Hasan 1994). This suggests that other gibberellin biosynthesis inhibitors with lower residual activity might be as effective and more attractive than paclobutrazol for general use. Chapter 4 investigates the affect of applying two alternative gibberellin biosynthesis inhibitors, chlormequat chloride and prohexadione to 11 month old *E. nitens* seedlings on GA levels, precocious reproductive development and growth and compares them to paclobutrazol.

Apart from hormonal manipulation, there are other silvicultural treatments which can promote precocious and abundant flowering in trees. Treatments to accelerate growth in conifers can reduce the time from planting to first seed crop (Chalupka and Cecich 1997) and increase seed production (Griffin *et al.* 1984). Earlier and more abundant flowering in trees of *E. regnans* has been achieved by accelerating growth through the application of fertiliser (Cameron and Kube 1983). However, some environments conducive to good growth of *E. nitens* result in poor flowering (Eldridge and Griffin 1990). In conifers, a combined treatment of fertilisation and hormone manipulation has vastly improved flowering over what could be achieved if only one of the treatments was used (Daoudi *et al.* 1994). Chapter 5 begins with an investigation of young *E. nitens* nitrogen and phosphorus fertiliser trials and examines if these treatments affect the timing of either reproductive or vegetative maturity and if these events are related to tree size. This is followed with an approach to improve both precocious and abundant flowering by a

combined treatment with nitrogen fertiliser and paclobutrazol on juvenile (< 4 years old) and mature (> 4 years old) trees, and whilst there are concerns with the use of paclobutrazol, its effectiveness cannot be disputed.

Once the orchard is well located, well spaced and appropriately treated with nutrients and hormone regulators and is producing vast quantities of open pollinated seed it may well be time to consider the next generation of selections for orchard production. The only reliable way to produce seed of a guaranteed pedigree is through controlled pollination and this is most critical in the production of superior genotypes. The traditional method of controlled crossing in eucalypts requires three visits to the female tree for: (1) emasculation and flower isolation, (2) pollination at peak stigma receptivity and (3) de-isolation of the fertilised flower. Chapter 6 tests alternative pollination and isolation techniques which could potentially reduce the number of visits to the flower from three to one. These techniques are applied to both *E. nitens* and *E. globulus* and their success would mean a significant reduction in the cost of pedigree seed production and open the way for mass pollination.

This thesis takes a broad investigative sweep through issues affecting flowering and seed production in *E. nitens* with the particular aim of improving the commercial productivity of seed orchards. With the median price for a kilogram of cleaned *E. nitens* seed currently around $25,000, even small gains in productivity can have a substantial financial benefit.

Chapter 2

Macroenvironmental effects on flowering and seed production

2.1 INTRODUCTION

The commercial requirement of seed production is the antithesis of the natural production system. It is highly desirable that commercial quantities of genetically improved seed be produced as abundantly, cheaply and reliably as possible soon after it is developed from breeding programs. In reality, heavy seed crops are produced infrequently (anywhere from 1-4 years), after an extended (4 to 10 years) non-reproductive juvenile phase in canopies meters off the ground which by then are difficult to access (Moncur 1994).

Long term flowering patterns of eucalypts can be asynchronous between and within years (Ashton 1975, Loneragan 1979, Griffin 1980, Yates *et al.* 1994, Setterfield and Williams 1996, Pook *et al.* 1997). Within a site there can be both heavy and light flowering of adjacent trees in any year with uniform flowering occurring only when there is a coincidence of heavy flowering cycles across the population (Ashton 1975). Within a site and any flowering season, there can be a great deal of separation between flower opening dates between trees (Ashton 1975, Griffin 1980). Asynchronous flowering in an orchard leads to an imbalance in the genetic contributions (Burczyk and Chalupka 1997), reduction in genetic diversity (El-Kassaby 1995) and unpredictable genetic gain (Griffin 1982a) in the progeny.

A number of silvicultural techniques to shorten the inter-generational period and increase flowering regularity and abundance have been tested and had varying degrees of success on *E. nitens*. Techniques to reduce the inter-generational period by inducing precocious flowering include treatment of seedlings with gibberellin biosynthesis inhibitors either alone (Griffin *et al.* 1993, Moncur 1998, Chapter 4) or with fertiliser (Chapter 5). Similar techniques have also been used to promote regular and abundant flowering (Griffin *et al.* 1993, Moncur and Hasan 1994, Swain and Chiappero 1998, Chapter 5) and efforts have also included growth in espaliers (Moncur *et al.* 1994b), and induction of water stress (Moncur 1998). However, most cases have suffered from the vagaries of an environmental component often identified as temperature (Moncur *et al.* 1994b, Swain and Chiappero 1998), the significance of which was highlighted in Moncur and Hasan (1994). To overcome this, Moncur (1998) suggests grafted material in pots be moved between environments to optimise flower and seed production. However, the system would only suit small trees and is not suitable for large scale seed production. The alternative is to carefully locate the orchard in an area where the environment is favourable to seed production or one which can be manipulated favourably and cost effectively.

In a survey of 23 sites across Tasmania with altitudes ranging from 40 to 720 metres above sea level (masl), Moncur *et al.* (1994a) identified site characteristics which seemed to favour greater flower and seed production. These desirable sites were generally warmer with a drier winter and promoted a longer flowering period. This survey was carried out over 2 consecutive flowering years, the second year having a higher proportion of trees surveyed flowering than the first and it was suggested some trees still had yet to reach reproductive maturity (average age was 7.5 years). There was also concern expressed over a small number of extremely heavy flowering events at one site. Tests on seeds were carried out to examine the timing of maturity and these found a trend of delayed maturity with increasing altitude.

There has been considerable study done on maternal environment effects on seed quality and progeny performance in many species (Roach & Wulff 1987), including gymnosperm forest tree species (Skrøppa 1994, Andersson 1994, Lindgren and Wei 1994, Johnsen *et al.* 1995, Schmidtling 1996). The maternal environment can not only affect the number and size of seeds, but seed germination capacity and rate (Roach & Wulff 1987). Indeed, evidence of a maternal environment after-effect has been found in trees of *Pinus* species of 5-6 years of age (Lindgren and Wei 1994, Schmidtling 1996). Commercially, this could affect the seed producer, nursery production system and plantation management.

This chapter revisits four sites studied by Moncur *et al.* (1994a) which were along an altitudinal transect, covering most of the range in that study (60 to 650 masl), four and five years after their second census. This new sample would allow for a comparison of age and size effects on the reliability of flowering. The effect of water availability alluded to in Moncur *et al.* (1994a) is also examined more intensively by studying an established and intensively managed *E. nitens* water relations trial. The effect of these environments on reproduction was studied on three levels. Firstly, the effect on flower and capsule production and flowering synchrony was studied. Secondly, the effects on seed production, number of seeds per capsule and individual seed weight were assessed. Finally, an assessment of seed germination performance was made on seeds collected from the altitude transect and irrigation trial to examine if these maternal environments (different altitude or drought stress) significantly affected the viability and germination rate of the seeds imbibed at low temperature or depressed osmotic potential respectively.

2.2 METHODS AND MATERIALS

2.2.1 The effect of water stress on flowering and seed production

2.2.1.1 Site description

This study was conducted on an existing eucalypt water relations trial established by the CSIRO Division of Forestry (now CSIRO Forestry and Forest Products) in 1990. The trial design, management and measurement techniques are extensively detailed in Honeysett *et al.* (1996), White *et al.* (1998) and White *et al.* (1999) and will only be briefly described here. The trial consists of a 2 ha plantation established on an ex-pasture site in a low rainfall area of south-eastern Tasmania (Lat 42°49'S Long 147°36'E), close to Lewisham and approximately 9 km East of Hobart Airport (Honeysett *et al.* 1996) (Table 2.1).

Table 2.1 Meteorological data for 1995 from 1998 from Hobart Airport 9 km west of the Lewisham irrigation trial (supplied by the Commonwealth Bureau of Meteorology).

Hobart Airport 42°50'24"S 147°30'00"E 4 masl	**Mean daily minimum temperature (°C)**	**Mean daily maximum temperature (°C)**	**Annual total precipitation (mm)**	**Annual total evaporation (mm)**
1995	7.90	16.60	631.00	1229.10
1996	7.70	16.50	563.40	1187.90
1997	7.50	17.30	449.50	1262.00
1998	8.20	17.80	503.00	1403.60

The site was planted in August 1990 with seedlings from Forest Resources improved seed of juvenile persistent *E. nitens* in blocks of 11 rows of 19 trees in 6 replicates at an inter-row spacing of 3.5 m and within row spacing of 2 m. Within each block, a study sub-plot of five rows by six trees was established. The blocks were randomised with block plantings of a second seed lot of *E. nitens* (Barrington Tops) and two provenances of *E. globulus*. The site was intensively managed to exclude weed competition and predators. The automatic microsprinkler irrigation system was installed in October 1991 with three replicates irrigated and three replicates rainfed. Irrigation was supplied to maintain a soil water deficit in the irrigated replicates of between 20 and 40 mm. Supplementary irrigation was given to the rain fed replicates to avoid severe water stress. The three irrigated replicates (replicates 1-3) were located adjacent and down slope from the rainfed replicates (replicates 4-6) to avoid lateral movement of irrigation water into the rainfed replicates (Honeysett *et al.* 1996). In 1995, the two down-slope rainfed replicates (replicates 4 and 5) began receiving a cyclic regime of a water stress phase followed by an irrigation phase to avoid the next droughting event (semi-irrigation) and these phases were alternated across the two replicates with each period of droughting. A stress cycle was defined as a period when the pre-dawn leaf water potential in the rainfed treatments was significantly lower that that of the irrigated treatment (Honeysett *et al.* 1996). Measurements of soil water through the profile was made approximately twice monthly using a neutron moisture meter whilst pre dawn leaf water potential was measured approximately bi-monthly using a pressure chamber (Table 2.2). Height and DBH measurements were made annually on trees in the study sub-plots (Honeysett *et al.* 1996).

Table 2.2 Annual average soil water deficit and pre-dawn leaf water potential for 1995 to 1998 of the treatments in the irrigation trial located near Lewisham in south-eastern Tasmania (data supplied by D. Worledge CSIRO Forestry and Forest Products).

Year		Replicates 1-3 irrigated	Replicate 4 semi-irrigated	Replicate 5 semi-irrigated	Replicate 6 rainfed
1995	Average soil water deficit (mm) at 120mm	-28.91	-38.15	-41.34	-50.08
	Average pre-dawn leaf water potential (MPa)	.	.	.	.
1996	Average soil water deficit (mm) at 120mm	-29.64	-31.92	-39.74	-66.87
	Average pre-dawn leaf water potential (MPa)	0.40	0.76	1.14	1.64
1997	Average soil water deficit (mm) at 120mm	-9.95	-30.05	-47.92	-80.16
	Average pre-dawn leaf water potential (MPa)	0.41	0.73	0.82	1.77
1998	Average soil water deficit (mm) at 120mm	-16.98	-40.96	-53.03	-85.11
	Average pre-dawn leaf water potential (MPa)	0.32	0.35	1.52	1.36

2.2.1.2 Flowering phenology

The flowering phenology was assessed by using traps to sample the litter fall from the canopy analogous to those used by Ashton (1975). On the 30th of November 1996, 15 litter traps were erected across five of the replicates, one in replicate 1, two in each of replicates 2 and 3 and five in each of replicates 4 and 6. The traps were constructed from a square of Weathashade® 70% shade cloth suspended from an 80 cm galvanised steel post at each corner and covered an area of 3.24 m^2 (Fig. 2.1). Traps were weighted in the centre, forming a shallow funnel to prevent trap contents being blown out. The traps were placed randomly, whilst avoiding placement directly over a microsprinkler, in the periphery trees surrounding the study sub-plots but no closer than two rows from the outside of the block. The traps were orientated such that each corner was as close as possible to a tree trunk and with sufficient ground clearance not to interfere with the spray pattern of the micro-sprinklers. Litter was collected from each trap weekly in December to March inclusively and fortnightly in the other months. Litter from each trap and collection was air dried at room temperature in open paper bags. The dried contents in each bag were hand sorted and the number of (i) immature umbels (still enclosed in bracts), (ii) immature flower buds (operculum attached), (iii) opercula, (iv) open flowers/immature capsules (operculum shed but valves still closed) and (v) mature capsules (valves open) were counted. From this data not only were specific events and temporal changes in reproductive development traced but a flower bud and capsule budget could be produced (Ashton 1975).

Figure 2.1 Litter traps constructed from knitted nylon mesh suspended from steel droppers designed to collect flower parts as they fall from the canopy. The surface area of the trap = 3.24 m^2.

2.2.1.3 Seed collection

In December 1997 mature capsules were collected from 12 to 14 periphery trees in each treatment regime (irrigated, semi-irrigated, rainfed) none of which were in the outside rows of the block or surrounding the traps. Once trees with mature capsules were identified, trees were then selected for harvesting based on the accessibility of umbels for collecting by cutting away umbel bearing branchlets using a telescopic pruner. For each tree, umbels were removed from branchlets and grouped according to the number of capsules in the umbel (1-7). The capsules in each umbel size class were dried at room temperature to facilitate seed release. The seed was cleaned of chaff, sorted according to viability and insect damage (Hardner and Potts 1995) and counted to give an average number of seeds per capsule and umbel size class. The healthy viable seed in each umbel

class from each tree was bulked together and weight to obtain an average individual seed weight.

2.2.1.4 Seed Germination

To examine if the water limited maternal environment had affected the response of seeds to imbibition under conditions of depressed osmotic potential, germination of the seeds was carried out using a solution of polyethylene glycol (PEG). The g/g weight ratio of PEG 8000 to water required to make a solution of a nominal water potential (Ψ) of -0.2 MPa at 20°C was calculated using the formula:

$$[PEG] = (4 - (5.16\,\Psi T - 560\Psi + 16)^{0.5}) / (2.58T - 280) \quad \text{.....[Eqn. 2.1 (Michel 1983)]}$$

where Ψ = water potential in bars (1 bar = 0.1 MPa)

T = temperature in °C

A germination substrate was prepared by filling a 9 cm diameter disposable petri dish with 3g vermiculite covered with a single layer of Whatman No 1 filter paper. The seeds of six trees from each treatment regime (irrigated, semi-irrigated and rainfed) were used to assess germination characteristics. For each tree, 15 seeds were placed into each of 5 petri dishes containing the germination substrate moistened with 15 ml of distilled water and each of a further 5 petri dishes containing the substrate moistened with 15 ml of the PEG solution. Each dish was then given a light spray of 1 $g.L^{-1}$ benlate fungicide solution. The dishes were placed in a growth cabinet at a constant 20°C with 12 hours of light supplied by

fluorescent tubes at a photo flux density of 100 $\mu mol.m^{-2}.s^{-1}$ and arranged in 5 completely randomised replicates. The seeds from each tree were thus present in each replicate twice, once imbibing in distilled water and once imbibing in PEG solution.

Dishes were examined every two to three days for germination. Seeds where the radicle had ruptured the testa were considered to have germinated and were removed. To prevent desiccation, distilled water was added to dishes to maintain 1 to 2 ml of free water in the base of the dish. Scoring was maintained until germination had ceased, typically one week without germination (Battaglia 1993).

2.2.1.5 Statistical analysis

For statistical analysis, the appropriate data was transformed as necessary to optimise the normality of the residuals and homogeneity of the variances. All results from transformed data were back transformed for presentation. For each trait, specific *a priori* contrasts between the treatments were undertaken using Tukeys adjustment and least squares means and standard errors of the treatment effects calculated. The statistical models were initially fitted with the PROC GLM procedure in SAS (SAS 1992) to examine the need to transform the raw data. The models were then fitted again to the data (which was transformed as necessary) using the PROC MIXED procedure in SAS (SAS 1992) to generate the specific contrasts, least squares means and standard errors and fit covariates as necessary.

Growth measurements

For growth, the statistical model fitted based on single tree cross sectional area at breast height (CSA) was:

CSA = mean + treat + error .....(Eqn. 2.2),

where treat is the fixed effect of irrigation treatment regime (irrigated, semi-irrigated, rainfed).

Flowering phenology

In Tasmania *E. nitens* normally commences flowering in mid-summer (Tibbits 1989) and for this experiment a datum was established to provide a temporal reference point for flowering events (Griffin 1980). The datum was assigned to the first week in which the litter was collected in the first season (the first week in December 1996) and was designated flowering week 1. All events were timed from week 1 through to week 52 (last week in November 1997), the end of the first flowering year, after which the second flowering year was started at week 1 (first week in December 1997) and finished at week 52 (last week in November 1998). For calculation of flowering synchrony, a cut off point for the collection of opercula was used and defined as 2% of the total number of opercula collected in a given year. This was done to reduce the effect of opercula caught in the aerial litter (Wright and Calderon 1995).

A statistical model was fitted to the data for: (i) the week in which flowering commenced, (ii) flowering peaked, (iii) the flowering synchrony [quantified as the standard deviation of

flowering date Gorchov (1990)], (iv) total flowers (based on opercula), (v) pre-flowering buds lost annually, (vi) proportion of pre-flowering buds lost, (vii) proportion of immature capsules and (viii) total mature capsules of undetermined age, from each irrigation treatment regime (irrigated, semi-irrigated and rainfed) based on individual trap data for each flowering year. The model fitted was:

trait = mean + year + treat + treat*year + trap(treat) + error .....(Eqn. 2.3),

where year is the fixed effect of flowering year (1 or 2), treat is the fixed effect of the irrigation treatment regime (irrigated, semi-irrigated, rainfed), treat*year is the fixed two way interaction between irrigation treatment and flowering year and trap(treat) is the random effect of the trap (1-5) within a treatment regime. The trap(treat) effect was used to obtain an approximate test of the significance of the treatment effect, whereas the significance of the year and treat*year effects were tested against the error.

Seed Collection

A statistical model was fitted to the data in each umbel size class for the average number of viable seeds per capsule and average viable seed weight for each tree harvested from the three irrigation treatment regimes. The model fitted was:

trait = mean + umbel + treat + umbel*treat + tree(treat) + error.....(Eqn. 2.4),

where umbel is the fixed effect of the umbel size class (1-7), treat is the fixed effect of the irrigation treatment regime (irrigated, semi-irrigated, rainfed), umbel*treat is the fixed two

way interaction between umbel size and irrigation treatment and tree(treat) is the random effect of the tree within an irrigation treatment. The tree(treat) effect was used to obtain an approximate test of the significance of the treatment effect whilst the significance of all other terms was tested against the error. Analysis was also undertaken to examine and remove the effect of the number of viable seeds per capsule on seed weight by including the number of viable seeds per capsule as a covariate in Eqn. 2.4. Pearsons correlations between viable seeds per capsule and seed weight were carried out using PROC CORR procedure in SAS (SAS 1992) for all seeds collected and for seeds collected in each treatment.

Seed Germination

The rate of germination for each dish was determined by calculating the time taken from the commencement of imbibition to reach 50% of the final cumulative germination (t_{50}). A value for (t_{50}) was calculated by regressing probit transformed percentage germination data against the natural logarithm of elapsed time (Battaglia 1993).

A statistical model was fitted to the data for the rate of germination (t_{50}) and proportion of seeds which germinated for each dish. The model fitted was:

trait = mean + rep + Ψ + treat + Ψ*treat + tree(treat) + tree(treat)* Ψ + error....(Eqn. 2.5),

where rep is the random effect of replicate (1-5), Ψ is the fixed effect of the applied water potential (0 or -0.2 MPa), treat is the fixed effect of the irrigation treatment regime (irrigated, semi-irrigated, rainfed), Ψ*treat is the fixed interaction between the applied

water potential and the irrigation treatment, tree(treat) is the random effect of tree within irrigation treatment and tree(treat)* Ψ is the random interaction between tree within an irrigation treatment and the applied water potential. The tree(treat) effect was used to obtain an approximate test of the significance of the treat effect. The tree(treat)* Ψ effect was used to obtain an approximate test of the significance of the tree(treat), Ψ*treat and Ψ effects, whilst the significance of the tree(treat)* Ψ and rep terms were tested against the error. The effect of seed weight was examined and removed by inclusion of tree means for individual seed weight as a covariate in Eqn. 2.5.

2.2.2 The effect of altitude on flowering and seed production

2.2.2.1 Site descriptions

This study was conducted in a species and provenance growth trial established by the CSIRO Division of Forestry (now CSIRO Forestry and Forest Products) which consisted of four sites in an altitudinal transect at 60, 240, 440 and 650 masl in the Esperance Valley, south-eastern Tasmania (*ca.* Lat 43°15' S, Long 146°50' E) (Turnbull *et al.* 1993). The environmental characteristics, establishment and management of the sites are described in detail in Turnbull *et al.* (1993) and Beadle *et al.* (1996) and will only be briefly outlined here and in Table 2.3.

Table 2.3 Meteorological data for 1995 to 1998 from weather stations at Geeveston and Dover in south-eastern Tasmania (supplied by the Commonwealth Bureau of Meteorology).

Weather station location	Year	Mean daily minimum temperature (°C)	Mean daily maximum temperature (°C)	Annual total precipitation (mm)
Geeveston 43°10'00"S 146°55'03"E 60 masl	1995	5.00	15.90	999.00
	1996	5.30	15.90	1164.90
	1997	5.90	16.50	878.20
	1998	6.30	16.70	754.00
Dover 43°18'53"S 147°01'01"E 16 masl	1995	6.20	15.60	1060.80
	1996	7.70	16.50	1071.80
	1997	7.50	17.30	877.80
	1998	8.20	17.80	744.20

After clearing, sites were planted in 1983 with 6 adjacent species blocks with a 2 x 2 metre spacing between trees. The *E. nitens* species block was planted as a 17 x 17 tree block with a central 11 x 11 tree measurement block. Half of the *E. nitens* species block was planted with seedlings of Errinundra provenance (now classified as *E. denticulata,* [Cook and Ladiges 1991]), the remaining half in which this study was undertaken consisted of trees of the Toorongo provenance of *E. nitens* CSIRO seed lot 13611. The sites were intensively managed in the first 4 years through the addition of fertiliser and suppression of competition and predation (Turnbull *et al.* 1993).

2.2.2.2 Growth measurement

In December 1998 all *E. nitens* trees at each site were measured for diameter over bark at breast height.

2.2.2.3 Flowering phenology

On the 1st of December 1996, 4 litter traps were erected in the *E. nitens* blocks at each of the four sites. The traps were randomly located under trees in the central measurement block. The traps were constructed (see Fig. 2.1) and collections were made at the same frequency for the same 24 month period as in section *2.2.1.2*. The maximum and minimum temperatures for each litter collection period at each site was also recorded (Table 2.4). The litter was dried, sorted and an account for the flowering phenology was made as described in section *2.2.1.2*.

Table 2.4 Mean annual minimum and maximum temperatures for December 1996 to November 1998 (based on weekly and fortnightly observations) for plantations along an altitudinal transect in south-eastern Tasmania.

Year	Site altitude (masl)	Mean minumum (°C)	Mean maximum (°C)
1996/97	60	2.29	22.01
	240	3.51	20.71
	440	0.85	18.85
	650	0.27	16.12
1997/98	60	0.81	22.38
	240	3.08	20.48
	440	0.53	18.18
	650	0.12	15.77

2.2.2.4 Seed Collection

In March 1998 mature capsules were collected from 9 to 11 *E. nitens* trees at each site. Trees were selected for harvest based on the number of accessible capsules for collecting by cutting away umbel bearing branchlets using secateurs on a telescopic pole. After their removal from the branchlets, the capsules were sorted and grouped by the number per umbel, dried to facilitate seed release, the seed was then cleaned, sorted, counted and weighed as in section *2.2.1.3*.

2.2.2.5 Seed Germination

Seeds from each of six trees from each site were used for germination testing. Germinations were carried out in petri dishes prepared as described in section *2.2.1.4* except seeds were imbibed with only distilled water. For each tree, 15 seeds were placed into each of 10 petri dishes containing the moistened germination substrate. The petri dishes were placed in growth cabinets and arranged randomly in five replicates of one dish per tree in each replicate in each of two cabinets. Conditions in the first cabinet were as described in section *2.2.1.4* whilst conditions in the second cabinet were identical to the first cabinet except the temperature was maintained at a constant 12°C instead of 20°C. Dishes were scored for germination and maintained as described in section *2.2.1.4*.

2.2.2.6 Statistical analysis

Data from the flowering phenological study, seed collection, seed germination and growth was analysed as described in section *2.2.1.5* with only minor modifications to the statistical models. In the flowering phenological, seed collection and growth studies, irrigation treatment regime (irrigated, semi-irrigated, rainfed) was replaced with site altitude (60, 240, 440 and 650) and trap number was reduced from 5 to 4 where appropriate in Eqns. 2.2, 2.3 and 2.4. To test the effect of tree size on reproductive abundance, on its own and with site altitude, over the altitudinal transect, the mean cross sectional area measured in 1998 of those trees immediately surrounding each trap was included as a covariate in Eqn. 2.3 for both flowering years.

In the seed germination study, Eqn. 2.5 was modified to:

trait = mean + rep(temp) + temp + alt + temp*alt + tree(alt) + tree(alt)*temp + error....(Eqn. 2.5a),

where rep(temp) is the random effect of replicate within germination temperature (1-5), temp is the fixed effect of germination temperature (12 or 20°C), alt is the fixed effect of the altitude of the site from which seed was harvested (60, 240, 440 and 650), temp*alt is the fixed two way interaction of germination temperature and site altitude from which seed was harvested, tree(alt) is the random effect of the tree (1-6) at an altitude and tree(alt)*temp is the random interaction between tree at an altitude and germination temperature. The rep(temp) and tree(alt) effects were used to obtain an approximate test of the significance of the effect of germination temperature and site altitude respectively. The tree(alt)*temp effect was used to obtain an approximate test of the significance of the effect of germination temperature, the interaction between germination temperature and site altitude, and each tree at each site altitude. All other terms were tested against the error.

2.3 RESULTS

2.3.1 The effect of water stress on flowering and seed production

2.3.1.1 Flowering phenology and growth

The total number of flowers which opened in both years was significantly affected by the level of water available to the tree (Table 2.5). The most abundant flowering in both years occurred under conditions where water availability was at its lowest whilst flowering was

significantly reduced by subjecting trees to a cyclic regime of water stress and irrigation (Figure 2.2). The number of flowers produced appears to be limited by the number of buds initiated rather than the rate of bud abortion between initiation and flower opening as there was no significant difference between treatments in the proportion of pre-flowering buds lost in either year (Table 2.5). The result for the proportion of pre-flowering buds lost is a robust estimate despite the buds being of indeterminate age as there was no significant variation in the number of pre-flowering or total flowers between years (Table 2.5).

There was no significant difference in tree cross sectional area between the rainfed and semi-irrigated treatments ($p > 0.05$) despite the significant difference in flower abundance (Table 2.5). In contrast, the fully irrigated trees had significantly greater stem cross sectional areas than those in the rainfed or semi-irrigated treatments ($p < 0.001$) but produced flowers at a rate which was intermediate to both and not significantly different to either (Figure 2.2). This suggests tree size in this case is not a major controlling effect on flower abundance. The number of mature capsules collected was not significantly affected by water availability in either year, and did not differ significantly from year to year (Table 2.5).

There was no significant effect of water availability or year on the timing of either the commencement of flowering, the flowering peak or, the synchrony of flowering in the irrigation trial (Table 2.5). Flowering in both years generally commenced in mid January and peaked in late January to early February (Table 2.6).

Table 2.5 ANOVA table for the effects of year and irrigation treatment on the reproductive phenology of mature trees in a plantation situated in a low rainfall area (<500 mm.year^{-1}) of south-eastern Tasmania. Levels of significance are shown whilst F values are given for fixed effects and Z values are given for [a]random effects. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), * = $p < 0.05$.

Effect	*Year*	*Treatment*	*Year *Treatment*	*Trap (Treatment)* [a]
DF	1	2	2	12
Error DF	12	12	12	12
Pre-flowering buds lost annually	2.59 n.s.	1.46 n.s.	0.28 n.s.	1.27 n.s.
Proportion of pre-flowering buds lost	3.94 n.s.	0.21 n.s.	0.84 n.s.	0.98 n.s.
Total flowers	1.12 n.s.	5.97 *	0.16 n.s.	0.29 n.s.
Proportion of immature capsules lost	2.62 n.s.	0.42 n.s.	2.25 n.s.	0.57 n.s.
Mature capsules	2.88 n.s.	0.59 n.s.	0.03 n.s.	2.24 *
Week flowering commenced	2.31 n.s.	3.36 n.s.	1.73 n.s.	0.67 n.s.
Week flowering peaked	0.56 n.s.	1.82 n.s.	0.45 n.s.	. n.s.
Flowering synchrony	1.51 n.s.	0.25 n.s.	0.70 n.s.	1.00 n.s.

Table 2.6 The timing of flowering events of mature trees over two consecutive seasons in a plantation which is receiving different irrigation regimes and situated in a low rainfall area (<500 mm.year^{-1}) of south-eastern Tasmania. These events are based on litter trap means where the week 1 datum was the first week in December in both 1996 (first season) and 1997 (second season). Flowering synchrony expresses the standard deviation of the weeks in which flowers opened (Gorchov 1990).

Year	Irrigation regime	Week flowering started	Week flowering peaked	Flowering synchrony
1996/97	irrigated	5.80	9.30	1.47
	semi-irrigated	8.09	9.50	1.46
	rainfed	6.00	9.90	1.39
1997/98	irrigated	6.20	8.50	1.37
	semi-irrigated	6.44	8.83	1.08
	rainfed	5.20	10.20	1.42

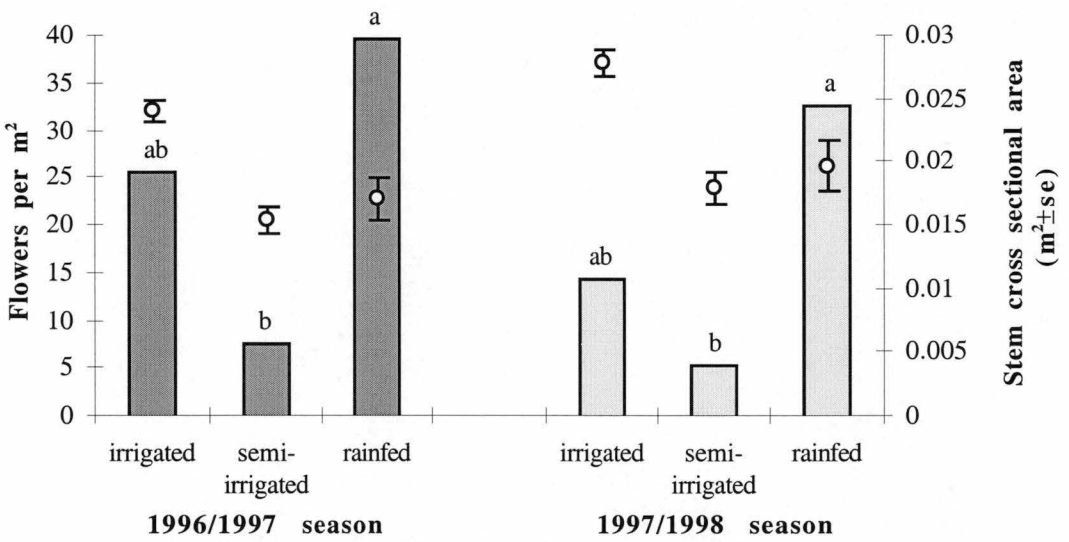


Figure 2.2 The number of flowers which opened (bars), based on opercula collected in litter traps over two seasons and mean tree cross sectional area at breast height (o ±se) in a plantation receiving different irrigation regimes and located in a low rainfall area (<500 mm.year^{-1}) of south-eastern Tasmania. The plantation was established in mid 1990 and irrigation treatments commenced in late 1991. Values for the number of flowers in the same season and with the same letter are not significantly different ($p > 0.05$). The effect of season was not significant ($p > 0.05$).

2.3.1.2 Seed quality

Seed production

The tree from which the seed was harvested had a highly significant affect both the number of seeds per capsule and individual seed weight (Table 2.7). The more seeds which developed in a capsule, the lighter their weight as individual seed mass was negatively correlated ($r = -0.31$) with the number of seeds per capsule. This negative effect on seed weight of the number of seeds per capsule was highly significant ($F = 62.03$, $p < 0.001$). However, the effect of the maternal tree on seed weight was not due to the number of seeds per capsule as the tree effect on seed weight remained strongly significant ($Z = 4.00$, $p < 0.001$) when the number of seeds per capsule was accounted for by covariate analysis.

The number of viable seeds per capsule and the seed weight was not significantly affected by the availability of water to the maternal parent (Table 2.7) and this is counter to the significant effects water availability had on growth and flowering. The mean numbers of seeds per capsule were 3.8, 4.3 and 3.4 from trees in the irrigated, semi-irrigated and rainfed treatments respectively. On a capsule per umbel basis, the mean number of seed set per capsule ranged from a minimum of 3.6 to a maximum of 4.0 in umbels with 2 and 5 capsules, respectively. The mean individual seed weights were 0.53, 0.47 and 0.49 mg from trees in the irrigated, semi-irrigated and rainfed treatments respectively. On a capsule per umbel basis, the mean individual seed weights ranged from a minimum of 0.49 mg to a maximum of 0.51 mg in umbels with 2 capsules and 1 capsule, respectively.

Table 2.7 ANOVA table for the effects of irrigation treatment and the number of capsules in an umbel on the characteristics of seeds collected from trees in a plantation situated in a low rainfall area (<500 mm.year^{-1}) of south-eastern Tasmania. Levels of significance are shown. F values are given for fixed effects whilst Z values are given for [a]random effects. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), *** = $p < 0.001$.

Effect	*Treatment*	*Capsules per umbel*	*Capsules per umbel *Treatment*	*Tree (Treatment)* [a]
DF	2	6	12	36
Error DF	36	177	177	177
Viable seeds per capsule	1.91 n.s.	0.63 n.s.	1.71 n.s.	3.76 ***
Individual seed weight	0.86 n.s.	0.47 n.s.	1.36 n.s.	3.95 ***

Seed germination

There was a significant reduction in both the success and rate of germination of seeds which were imbibed at a reduced water potential (Figures 2.3 and 2.4), whilst the maternal tree had a significant effect only on the rate of germination (Table 2.8). The water availability of the maternal environment did not significantly affect the success or rate of germination, nor was there a significant interaction between imbibing water potential and the water availability of the maternal environment or the maternal genotype (Table 2.8). When seed weight was accounted for, the significance of the main effects and their interactions remained unchanged whilst the effect of seed weight on the success and rate of germination was not significant ($p < 0.05$)

Table 2.8 ANOVA table for the effects of maternal tree irrigation treatment and the water potential during germination on the proportion and rate of germination of filled seeds harvested from trees under different irrigation regimes in a plantation situated in a low rainfall area (<500 mm.year^{-1}) of south-eastern Tasmania. Levels of significance are given whilst F valued are given for fixed effects and Z values are given for [a]random effects. Key: Ψ = water potential, DF = degrees of freedom, n.s. = not significant ($p > 0.05$), *** = $p < 0.001$.

Effect	*Treatment*	*Germination* Ψ	*Germination* Ψ * *Treatment*	*Tree (Treatment)* [a]	*Tree (Treatment)* *Germination Ψ [a]
DF	2	1	2	15	15
Error DF	15	15	15	15	140
Proportion of filled seeds which germinated	0.80 n.s.	44.64 ***	0.70 n.s.	1.82 n.s.	1.54 n.s.
Time required for cumulative germination to reach 50 %	0.01 n.s.	400.46 ***	1.54 n.s.	2.31 *	0.00 n.s.

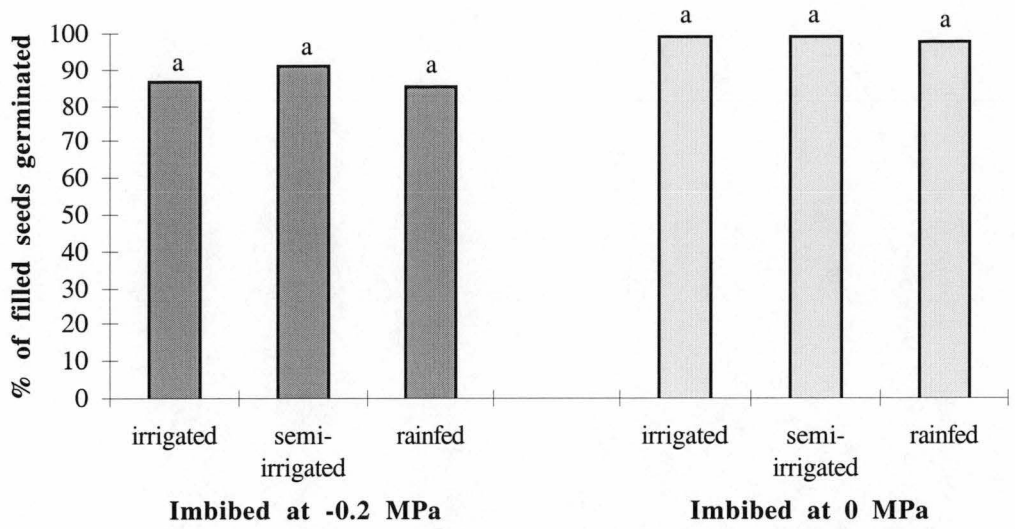


Figure 2.3 The percentage of filled seeds which germinated when imbibed at different water potentials. The seeds were harvested from trees receiving different irrigation regimes in a plantation situated in a low rainfall area (<500 mm.year^{-1}) of south-eastern Tasmania. Values with the same letter and within the same imbibing water potential are not significantly different ($p > 0.05$). The overall significance of the imbibing water potential effect on germination success was $p < 0.001$.

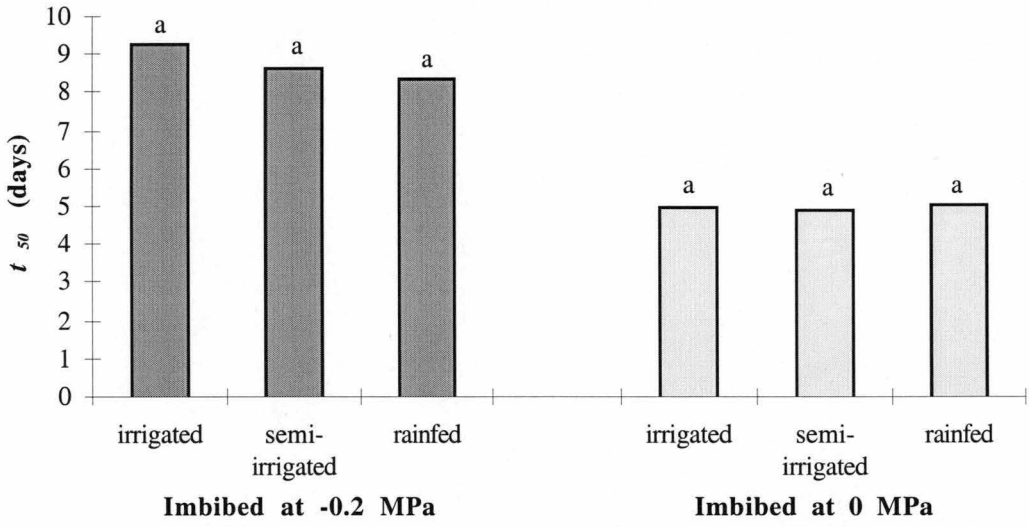


Figure 2.4 The time taken in days from the commencement of imbibition for seedlots to reach 50% of the final cumulative germination (t_{50}) when imbibed at different water potentials. The seeds were harvested from trees receiving different irrigation regimes in a plantation situated in a low rainfall area (<500 mm.year^{-1}) of south-eastern Tasmania. Values with the same letter and within the same imbibing water potential are not significantly different ($p > 0.05$). The overall significance of the imbibing water potential effect on germination rate = $p < 0.001$.

2.3.2 The effect of altitude on flowering and seed production

2.3.2.1 Flowering phenology and growth

There was a significant effect on both the start and peak of flowering as site altitude increased from 60 to 650 m (Table 2.9). The commencement of flowering was delayed by approximately 3 weeks whilst the peak of flowering was delayed by approximately 2 weeks at the 650 m site compared to the 60m site in both years (Table 2.10). Both the commencement and peak of flowering occurred significantly earlier in the second year compared to the first by about 1.5 weeks (Table 2.10). Flowering synchrony did not change significantly between years (Table 2.9) and was only affected by site in the first year where flowering at the 240 m site was significantly more asynchronous than flowering at both the 440 m and 650 m sites (Table 2.10).

The site of the trials significantly affected stem radial growth ($F = 5.29$, $p < 0.01$), flower abundance, the loss of unopened flowers and immature capsules and the fall of mature capsules (Table 2.9). However, there was no clear relationship between growth or reproductive traits and increasing site altitude (Figures 2.5 to 2.8). Site effect on tree survival could not be determined due to previous destructive sampling whilst the numbers of trees remaining at the 60 m, 240 m, 440 m, and 650 m sites were 44, 46, 49 and 44 respectively. The effect of site on reproductive output could not be attributed to tree size as when tree cross sectional area was accounted for, the site effects remained significant (Table 2.11). Flower abundance and mature capsule fall was greatest at the 60 m site and least at the 240 m site in both years whilst stem cross sectional area was greatest at the 650 m site (Figures 2.5 and 2.8).

Table 2.9 ANOVA table for the effect of year and site on the reproductive phenology of mature trees in trials along an altitudinal transect in south-eastern Tasmania. Levels of significance are shown whilst F values are given for fixed effects and Z values are given for [a]random effects. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Effect	*Year*	*Site*	*Year*Site*	*Trap(Site)* [a]
DF	1	3	3	12
Error DF	12	12	12	12
Pre-flowering buds lost annually	3.23 n.s.	16.88 ***	0.84 n.s.	1.36 n.s.
Proportion of pre-flowering buds lost	28.14 ***	13.18 ***	18.16 ***	1.57 n.s.
Proportion of immature capsules lost	4.89 *	9.46 **	11.64 ***	1.33 n.s.
Mature capsules	11.88 **	8.26 **	5.30 *	2.27 *
Week flowering commenced	24.05 ***	10.21 **	0.49 n.s.	1.62 n.s.
Week flowering peaked	26.41 ***	17.44 ***	3.59 *	. n.s.
Flowering synchrony	1.83 n.s.	5.54 *	1.71 n.s.	. n.s.

The proportion of unopened flower buds which were lost was greatest at the 650 m site in both years, significantly so in the first year (Figure 2.6) and this may have contributed to the lower number of flowers which opened at this site in the first year compared to the second (Figure 2.5). Indeed the 1996/97 flowering season was particularly bad at this site as a substantially high proportion of the flowers which did open were lost before maturing into capsules (Figure 2.7). It is not clear if these losses were due to biotic or abiotic factors.

Table 2.10 The timing of flowering events over two years in plantations along an altitudinal transect in south-eastern Tasmania. The data is based on litter trap means where the week 1 datum was the first week in December in both 1996 (first season) and 1997 (second season). Values in each season and series with the same letter are not significantly different ($p > 0.05$).

Year	Site altitude (masl)	Week flowering started	Week flowering peaked	Flowering synchrony
	60	3.25 a	8.25 ab	1.43 ab
1996/97	240	6.00 b	8.00 a	1.83 a
	440	6.50 b	9.25 bc	1.11 b
	650	7.00 b	10.00 c	1.07 b
	60	2.25 a	6.00 a	1.17 a
1997/98	240	5.00 b	7.75 b	1.36 a
	440	5.00 b	8.50 b	1.27 a
	650	5.25b	8.50 b	1.04 a

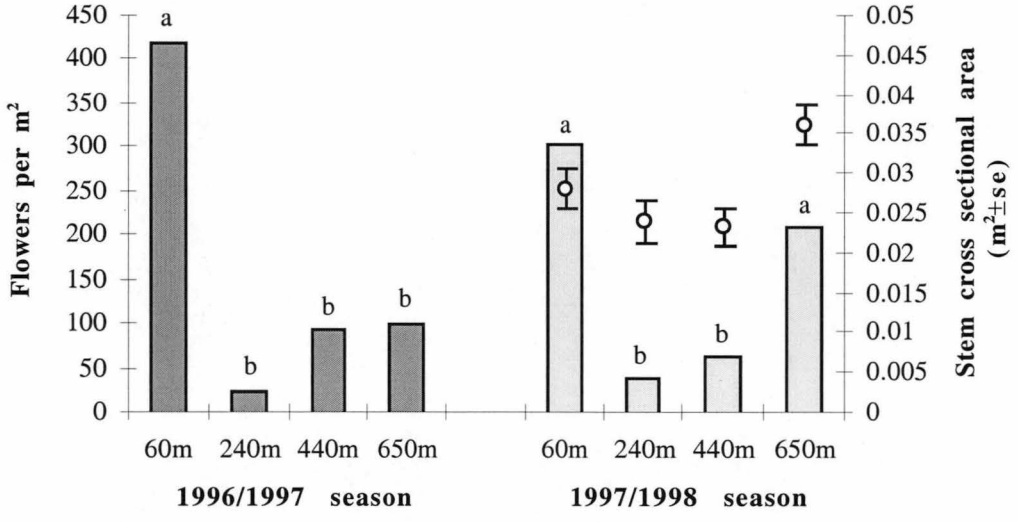


Figure 2.5 The number of flowers which opened (bars), based on opercula collected in litter traps, over two seasons and mean tree cross sectional area at breast height (o ±se) measured in December 1998 in trials along an altitudinal transect in south-eastern Tasmania. The trials were planted in late 1983 and respective altitudes in metres (m) are shown. Values for the number of flowers in the same season and with the same letter are not significantly different ($p > 0.05$). The effect of season on flowering was not significant ($p > 0.05$).

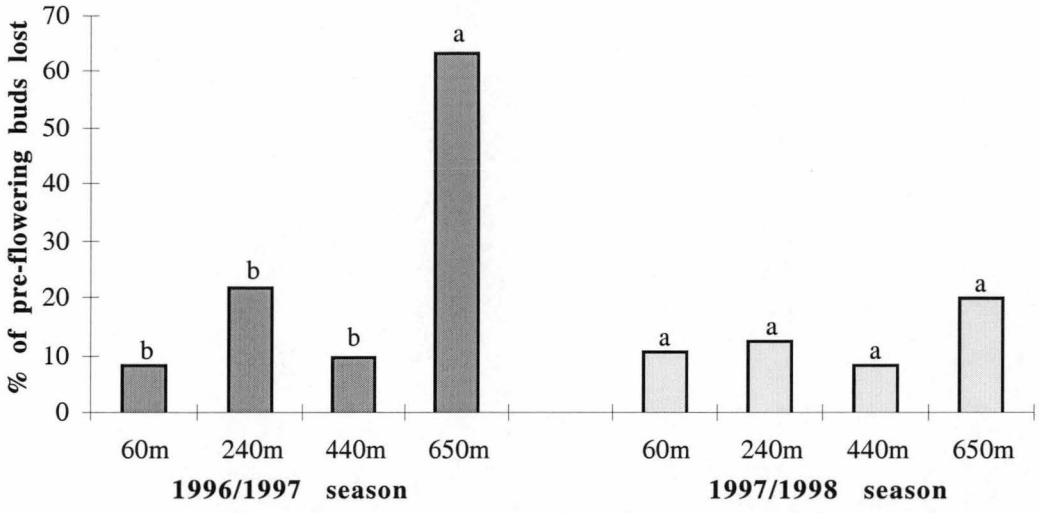


Figure 2.6 The proportion of flower buds aborted prior to opening based on litter trap collections made over two years in trials along an altitudinal transect in south-eastern Tasmania. The trials were planted in late 1983 and respective altitudes in metres (m) are shown. Values for the number of flower buds in the same season and with the same letter are not significantly different ($p > 0.05$). The effect of season on flowering was not significant ($p > 0.05$).

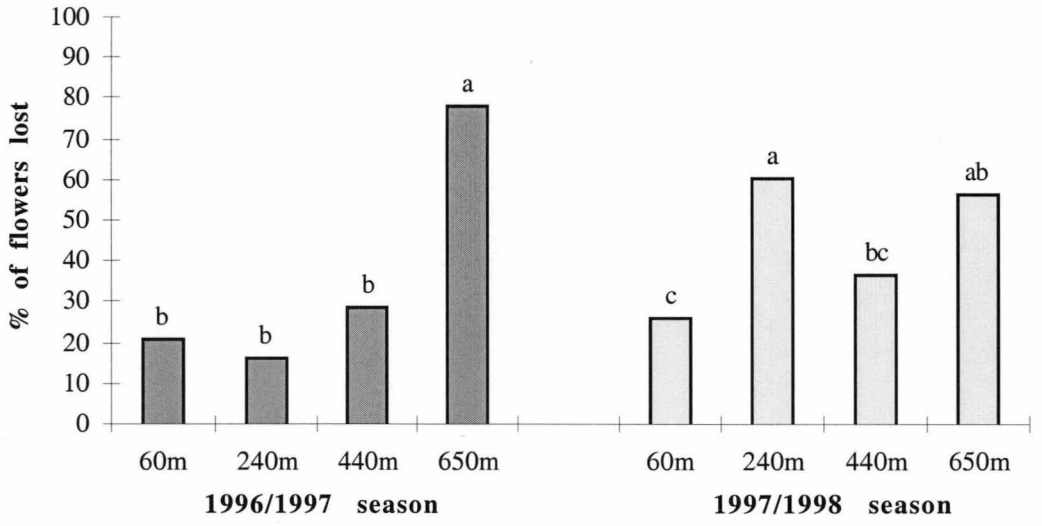


Figure 2.7 The percentage of flower buds which opened but were lost before maturing into capsules. The calculations were based on the ratio of the number of open flowers and immature capsules to the number of opercula which were collected in litter traps over two seasons in trials along an altitudinal transect in south-eastern Tasmania. The trials were planted in late 1983 and respective altitudes in metres (m) are shown. Values in the same season with the same letter are not significantly different ($p > 0.05$). The effect of season was significant ($p < 0.05$).

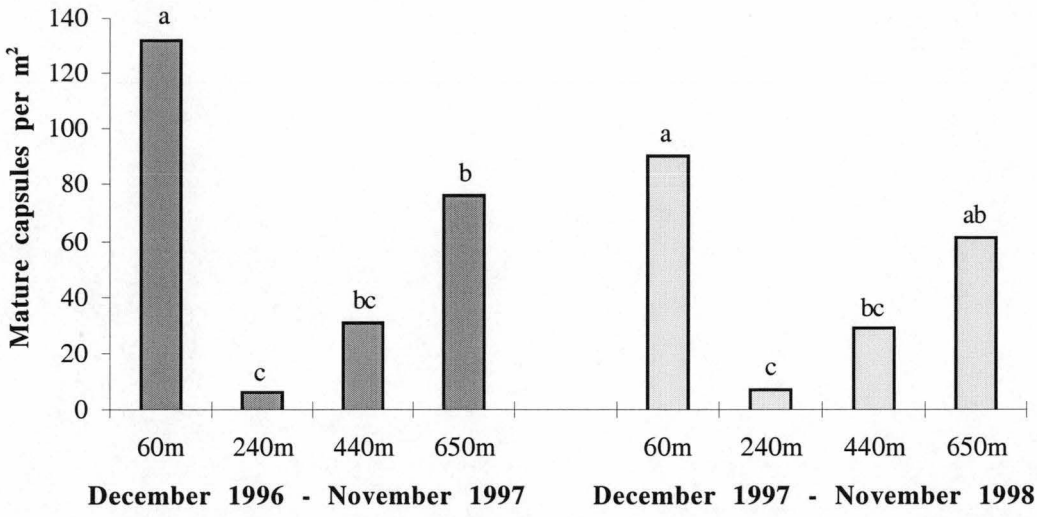


Figure 2.8 The number of mature capsules collected in litter traps over two seasons in trials along an altitudinal transect in south-eastern Tasmania. The trials were planted in late 1983 and the respective altitudes in metres (m) are shown. Values in the same season with the same letter are not significantly different ($p > 0.05$). The effect of season was significant ($p < 0.01$).

Table 2.11 ANOVA table for the effect of year and site, with tree cross sectional area included as a covariate, on the reproductive phenology of mature trees in trials along an altitudinal transect in south-eastern Tasmania. Levels of significance are shown whilst F values are given for fixed effects and Z values are given for [a]random effects. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Effect	*Covariate (Tree size)*	*Year*	*Site*	*Year*Site*	*Trap(Site)* [a]
DF	1	1	3	3	12
Error DF	12	12	11	12	12
Pre-flowering buds lost annually	0.62 n.s.	3.23 n.s.	10.77 **	0.84 n.s.	1.34 n.s.
Proportion of pre-flowering buds lost	0.27 n.s.	28.14 ***	7.97 **	18.16 ***	1.56 n.s.
Total flowers	0.68 n.s.	0.08 n.s.	13.13 ***	6.39 **	1.78 n.s.
Proportion of immature capsules lost	0.79 n.s.	4.89 *	8.33 **	11.64 ***	1.30 n.s.
Mature capsules	0.00 n.s.	11.88 **	7.14 **	5.30 *	2.19 *

2.3.2.2 Seed quality

Seed production

The number of seeds per capsule was significantly affected by number of capsules per umbel and the tree from which the seed was harvested (Table 2.12). The number of seeds per capsule increased by up to 25% as the number of capsules per umbel increased from 1 to 7 (Figure 2.9) and the effect was consistent across all sites. The number of seeds per capsule was not significantly affected by the site from which it was harvested. However

there was a trend for the number of seeds per capsule to decrease as site elevation increased (Figure 2.9).

Individual seed weight was significantly affected by the site and the tree from which it was harvested (Table 2.12). There was no direct relationship between seed weight and site altitude as seed weight was greatest at the 240 m site whilst the 650 m site produced the lightest seed (Figure 2.10). Individual seed weight was not significantly affected by the number of capsules per umbels (Table 2.12). This suggests the total mass of seed per capsule increases as the number of capsules per umbel increases. The significant tree effects on individual seed weight could not be accounted for by the number of seeds per capsule (Table 2.13). Nevertheless, neither the significant tree effect nor the site effect on seed weight was accounted for by variation in the number of seeds per capsule although the site effect was diminished

In general, individual seed weight was significantly decreased with increasing numbers of seed per capsule ($p < 0.001$) and the significant ($p < 0.05$) interaction between the covariate and site suggests the relationship differed between sites. The covariate partially accounted for some of the effects of site on individual seed weight as the level of significance of the site effect was reduced (Table 2.13). The interaction between the effects of site and the number of seeds per capsule were due to differences in the magnitude, direction and significance of their correlations between sites. Seed weight was negatively correlated to different degrees with the number of viable seeds per capsule at the three most elevated sites ($r = -0.18$ and $p > 0.05$ at 240 m, $r = -0.30$ and $p < 0.01$ at 480 m and $r = -0.24$ and $p < 0.05$ at 650 m), whilst at the 60 m site, seed weight was significantly ($p < 0.001$) and positively ($r = 0.61$) correlated with the number of viable seeds per capsule.

Table 2.12 ANOVA table for the effects of site, tree and the number of capsules in an umbel on the characteristics of seeds harvested from trees in trials situated along an altitudinal transect in south-eastern Tasmania. Levels of significance are shown whilst F valued are given for [a]random effects. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), *** = $p < 0.001$.

Effect	*Site*	*Capsules per umbel*	*Capsules per umbel*Site*	*Tree(Site)* [a]
DF	3	6	18	36
Error DF	36	205	205	205
Viable seeds per capsule	1.49 n.s.	11.29 ***	0.86 n.s.	4.08 ***
Individual seed weight	17.76 ***	1.13 n.s.	1.24 n.s.	3.83 ***

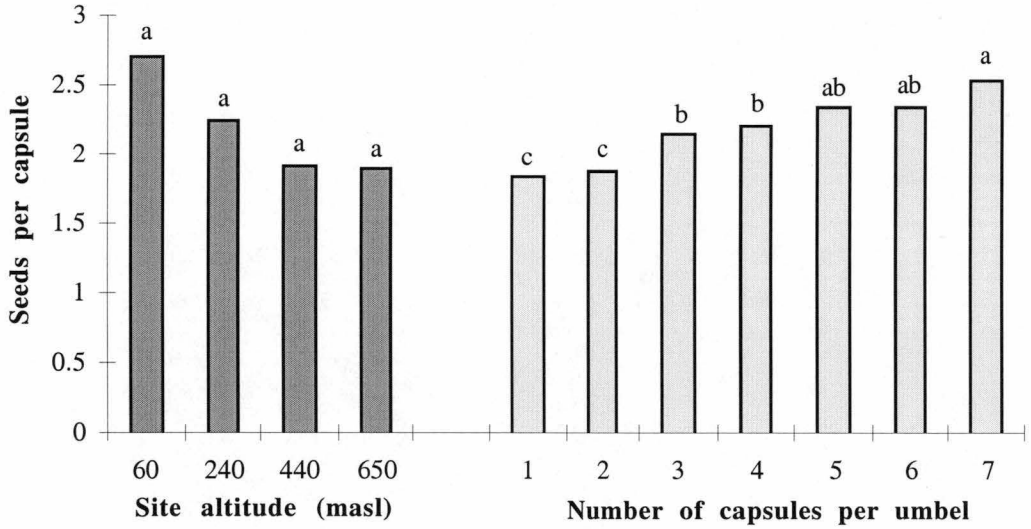


Figure 2.9 The mean number of viable seed per capsule from different sized umbels harvested from trial sites along an altitudinal transect in south-eastern Tasmania. Capsules were harvested in March 1998 dried at room temperature and the seeds were hand sorted. Values in the same series and with the same letter are not significantly different ($p > 0.05$).

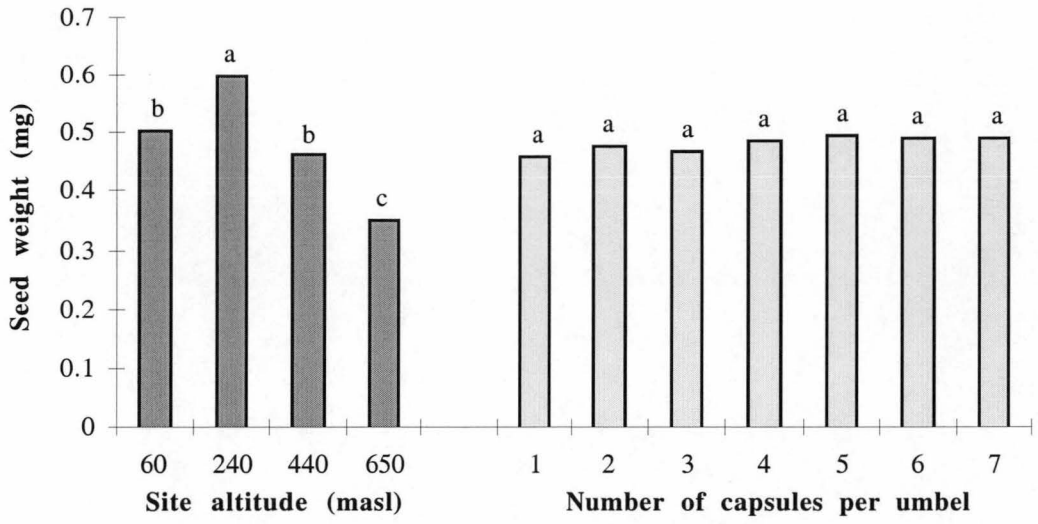


Figure 2.10 The mean individual weight of seeds from capsules in different sized umbels harvested from trees in trial sites along an altitudinal transect in south-eastern Tasmania. Capsules were harvested in March 1998 dried at room temperature and the seeds were hand sorted and weighed. Values in the same series and with the same letter are not significantly different ($p > 0.05$).

Table 2.13 ANOVA table for the effects of site and the number of capsules in an umbel, with the number of seeds per capsule included as a covariate, on the individual weight of seeds collected from trees in trials situated along an altitudinal transect in south-eastern Tasmania. Levels of significance are shown whilst F valued are given for fixed effects and Z values are given for [a]random effects. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), *** = $p < 0.001$.

Effect	*Covariate (Viable seeds per capsule)*	*Site* Covariate (Viable seeds per capsule)*	*Site*	*Capsules per umbel*	*Capsules per umbel*Site*	*Tree(Site)* [a]
DF	1	3	3	6	18	36
Error DF	201	201	36	201	201	201
Individual seed weight	11.33 ***	3.14 *	6.24 **	2.1 n.s.	1.19 n.s.	3.67 ***

Seed germination

The proportion of seeds which germinated and the rate of germination was significantly affected by the tree ($p < 0.05$), site ($p < 0.05$) and the temperature ($p < 0.001$) at which they were imbibed (Table 2.14). The proportion of seeds which germinated reduced as site elevation increased and this trend was present at both imbibition temperatures (Figure 2.11). The rate at which the seeds germinated was not directly related to the elevation of the site from which they were harvested with seeds from the 440 m site having the slowest rate of germination (Figure 2.12). The effect of site on the proportion of seeds which germinated and the rate of germination could not be explained by the differences in seed weight as its effect was not significant when included as a covariate (Table 2.15). However, there was a significant interaction between the maternal tree and the temperature at which seeds were imbibed on both the proportion of seeds which germinated and the rate of germination (Table 2.14) which could not be explained by seed weight (Table 2.15) and may indicate the optimum temperature for germination varies between trees.

Table 2.14 ANOVA table for the effects of maternal tree environment (site) and the temperature during germination on the proportion and rate of germination of filled seeds harvested from trees in trials along an altitudinal transect in south-eastern Tasmania. Levels of significance are given whilst F valued are given for fixed effects and Z values are given for [a]random effects. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), *** = $p < 0.001$.

Effect	*Site*	*Germination temperature*	*Germination temperature *Site*	*Tree (Site)* [a]	*Tree(Site) *Germination temperature* [a]
DF	3	1	3	20	20
Error DF	20	14	20	20	289
Proportion of filled seeds which germinated	4.46 *	91.00 ***	2.34 n.s.	2.39 *	2.70 **
Time required for cumulative germination to reach 50 %	3.61 *	240.77 ***	0.67 n.s.	2.39 *	2.23 *

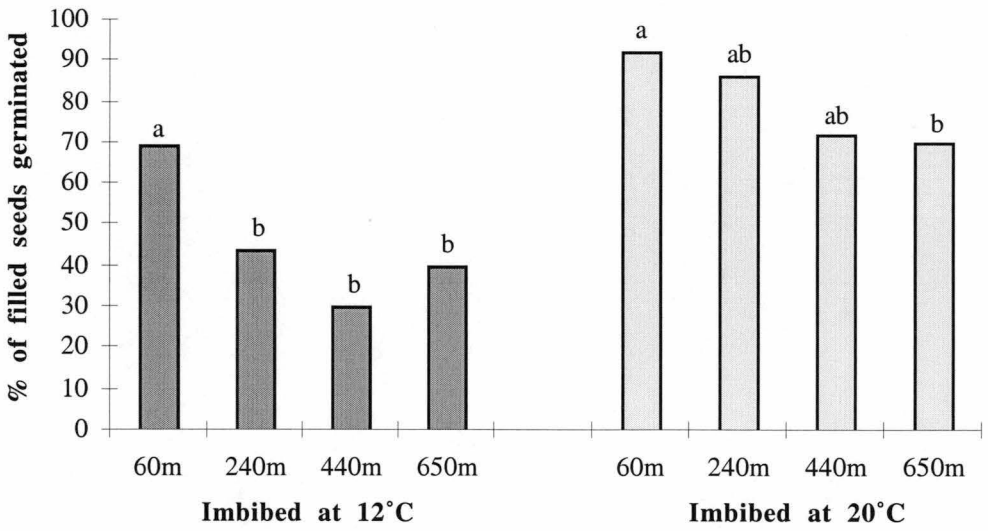


Figure 2.11 The percentage of filled seeds which germinated when imbibed at different temperatures with mean seed weight included as a covariate. The seeds were harvested from trees in trial sites along an altitudinal transect in south-eastern Tasmania and the respective altitudes in metres (m) are shown. Values with the same letter and the same germination temperature are not significantly different ($p > 0.05$). The effect of germination temperature was significant ($p < 0.001$).

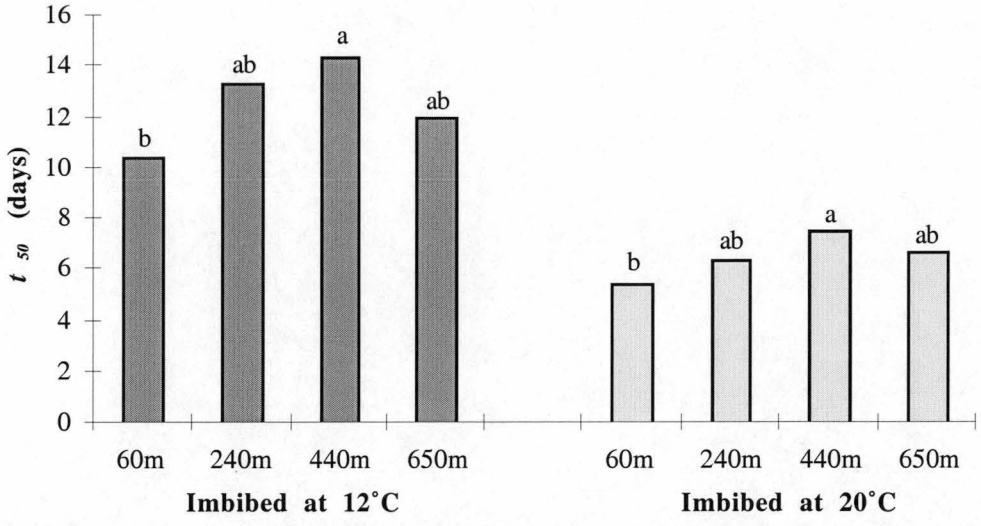


Figure 2.12 The time taken in days from the commencement of imbibition for seedlots to reach 50% of the final cumulative germination (t_{50}) when imbibed at different water potentials with mean seed weight included as a covariate. The seeds were harvested from trees in trial sites along an altitudinal transect in south-eastern Tasmania and the respective altitudes in metres (m) are shown. Values with the same letter and the same germination temperature are not significantly different ($p > 0.05$). The effect of germination temperature was significant ($p < 0.001$).

Table 2.15 ANOVA table for the effects of maternal tree environment (site) and the temperature during germination, with individual seed weight included as a covariate, on the proportion and rate of germination of filled seeds harvested from trees in trials along an altitudinal transect in south-eastern Tasmania. Levels of significance are given whilst F values are given for fixed effects and Z values are given for [a]random effects. Key: Ψ = water potential, DF = degrees of freedom, n.s. = not significant ($p > 0.05$), *** = $p < 0.001$. [b]The error degrees of freedom shown are for proportion of filled seeds which germinated/time required for cumulative germination to reach 50%.

Effect	*Covariate (Individual seed weight)*	*Site*	*Germination temperature*	*Germination temperature* Site*	*Tree(Site)* [a]	*Tree (Site)* Germination temperature* [a]
DF	1	3	1	3	20	20
Error DF	319/288[b]	20	14	20	20	289
Proportion of filled seeds which germinated	0.13 n.s.	3.06 n.s.	91.00 ***	2.34 n.s.	2.37 *	2.70 **
Time required for cumulative germination to reach 50%	0.68 n.s.	2.85 n.s.	240.97 ***	0.67 n.s.	2.34 *	2.23 *

2.4 DISCUSSION

Flower production in *E. nitens* is sensitive to water availability. Flowering abundance was greatest under conditions of drought stress in the irrigation trial. Drought stress has been reported to induced heavy flower crops in the following season in *E. viminalis* (Moncur 1998) and heavy seed crops in *E. diversicolor* (Eldridge *et al.* 1993). However, drought has also substantially reduced flower bud crops and subsequent seed production in *E. regnans* (Ashton 1975), *E. macrorhyncha* (Ashton and Sandiford 1988) and *E. maculata* (Pook *et al.* 1997). In contrast, drought stress broken intermittently by watering in the irrigation trial significantly depressed the flowering *E. nitens*. In a number of plant

species, the timing of water stress can be critical to the induction of flower buds (Jackson and Sweet 1972). In lemon (*Citrus limon*), flower abundance was increased as the period of water stress was increased from two to eight weeks whilst a cyclic regime of water stress was less effective (Chaikiattiyos *et al.* 1994). Intermittent drought stress of *E. nitens* may be responsible to some degree for the observed irregular flowering habit.

Mature capsule production (and potentially seed production) could be expected to be greatest in the rainfed, drought stressed treatment and least in the semi-irrigated treatment. The rate of loss (by abortion or damage) of opened and unopened flowers in the irrigation trial was not significantly affected by water availability and it is possible that drought stress did not affect maternal competence. However, uniform pollinator activity might also account for the lack of difference between treatments in the loss of opened flowers (House 1997). Although there was no significant difference in the number of mature capsules collected, these were of indeterminate age and the collection subject to fluctuations not only in production but loss from the canopy. The sensitivity of flowering and capsule production in *E. nitens* to intermittent drought stress would be cause for considerable concern if similar patterns were to occur in operational orchards. Griffin *et al.* (1984) recommended that *P. radiata* orchards be located on drier sites and have irrigation systems to permit a high level of control over water availability in order to increase the production of seed and the reliability of the crop.

Water availability is not a controlling factor in the timing and synchrony of flower opening in the irrigation trial despite the effects on flower abundance and tree growth (Table 2.5). Water availability can alter flowering patterns of different species of eucalypts to varying degrees. The flowering of *E. amygdalina* occurred earlier on drier sites compared to wetter sites whilst flowering of *E. risdonii* across similar sites was more temporally uniform (Potts and Reid 1985). The uniformity of flower opening across the irrigation

trial would perhaps be better explained by temperature as the trial only covered two hectares the differences in temperature across the site, if any, would be extremely small. Temperature is known to strongly affect flower development in *E. nitens* (Moncur and Hasan 1994). In *E. nitens*, Moncur *et al.* (1994a) found the accumulated heat sum from flower initiation to opening across a wide range of sites was relatively uniform compared to large differences in the actual time between these events.

In the altitudinal transect, the overall trend in the phenology results was that flowering commenced and peaked later, as site altitude increased (Table 2.10). This same trend was observed at these sites previously by Moncur *et al.* (1994a) and in natural stands of *E. regnans* (Ashton 1975, Griffin 1980) and agrees with the general heat sum model proposed by Moncur *et al.* (1994a). However, Moncur *et al.* (1994a) found the 240 m site flowered earlier than the other three sites in the 1991/92 season and classed it in a separate flowering group. As altitude in the transect increased the mean maximum temperatures decreased (Table 2.4) although the minimum temperatures did not follow this trend as the 60 m site is subject to cold air drainage (Beadle *et al.* 1996). If the level of cold air drainage in 1991 at this site was particularly severe it may have delayed flowering significantly and explain why flowering the following season occurred after that at the 240 m site in the observations by Moncur *et al.* (1994a).

Annual differences in climate may account for the significant differences between the 1996/97 and 1997/98 seasons in the commencement and peak of flowering (Table 2.9), both of which occurred earlier in the second season (Table 2.10). The climate in south-eastern Tasmania over that period became warmer (Table 2.3). As flowering is closely correlated to an accumulated heat sum (Moncur *et al.* 1994a) this increase in regional temperature may account for the advancement in the flowering time (Table 2.5).

There is a proportional relationship between the number of flowers and the period in which they open. In the altitudinal transect, there was little change in the values for synchrony (Table 2.10) in either year despite the significant difference in flowering abundance across the sites (Figure 2.5). Indeed, the range of synchrony values found along the transect also covered that found at the irrigation trial (Table 2.6) despite there being substantially fewer flowers (Figure 2.2). This suggests the relationship is quite robust. Overall, this pattern is similar to the findings of Griffin (1980) for *E. regnans* where the larger the flower crop the longer the flowering period and this may enhance the diversity of crosses and the occurrence of out-crossing. However, there still may be some significant effect of site conditions on pollen release and timing and period of stigma receptivity (Hodgson 1976, Griffin and Hand 1979) which is yet to be explored.

Flower abundance did not conform to the same trend in the altitudinal transect as flowering time. In 1997/98 season the most abundant flowering occurred at the two extreme sites. This would have also been the case for the 1996/97 season if the high rate of unopened flower abortion at the 650 m site had not occurred (Figure 2.6). Indeed 1996/97 was a bad season for reproductive growth at the 650 m site as open flower loss rates were also high. Savva *et al.* (1988) found pollinator activity reduced as altitude increased and House (1993) found reduced flower abortion rates correlated with increased pollinating insect activity in *E. stellulata*. However, these trends were not consistent with the results for the rate of flower loss in the altitudinal transect (Figure 2.7) and there may be further biotic or abiotic influences responsible. Drought stress is unlikely to have caused these high rates of loss at this site as there were no significant effects of drought stress found in the irrigation trial.

The combined effects of both climate and site quality may be responsible for the lack of a clear relationship between site altitude, growth rate and the production of flowers (Figure 2.5) and capsules (Figure 2.8). *Eucalyptus nitens* has a wide band of tolerance in its photosynthetic responses to changes in growth temperature (Battaglia *et al.* 1996) and over this altitude range the assimilate production and therefore growth rate may be limited very little by temperature across these sites. Prior to establishment in 1983, the 650 m site had the highest site index (i.e. mean dominant tree height at age 50 years) of the four sites (Turnbull *et al.* 1993) and during site preparation both the 240 m and 440 m sites were substantially degraded due to loss of surface soil (Anon. 1985) and this is consistent with the tree cross sectional area measurements taken in 1998 at age 16 (Figure 2.5). Indeed, the pattern of flowering also follows this trend, as nutrient availability, in particular nitrogen, can affect flowering (Chapter 5). Although these sites were fertilised for the first four years (Turnbull *et al.* 1993) the artificial effects of the fertiliser could be seen to have diminished at age eight (Beadle *et al.* 1996). However, growth alone, which appears to be indicative of site quality, could not account for the effect of site on the number of flowers or capsules (Table 2.11) despite evidence of significant effects at other sites (Chapter 5).

Sites which not only promote good growth rates but experience regular frosts would be expected to promote high rates of flowering in *E. nitens*. The most important component of climate to flower induction in *E. nitens* is an obligate requirement for a period of low temperature (Moncur and Hasan 1994). Frosts at the 240 m site were rare (Turnbull *et al.* 1993) and the minimum temperature was relatively high (Table 2.4) and flowering rates were less than 50 flowers.m^{-2} in both seasons (Figure 2.5). Furthermore in the irrigation trial which receives very few frosts (White *et al.* 1996), the highest level of flower abundance observed was 40 flowers.m^{-2} in 1996/97 (Figure 2.2). These cases are in contrast to the 60 m site which receives regular frosts (Turnbull *et al.* 1993) and had relatively low minimum temperatures (Table 2.4) the flower abundance was of the order of

300 - 430 flowers.m^{-2}. However, a caveat would be not to expose trees to conditions which might also cause high rates of flower bud loss seen at the 650 m site in 1996/97.

Pollination and fertilisation appear to be isolated from water stress, be it continuous or cyclic as the number of seeds per capsule was unaffected by treatment. The number of seeds per capsule (from 3.4 to 4.3) agrees with the mean found by Tibbits (1989), of 3.8 seed per open pollinated capsule, though lower than that found by Moncur *et al.* (1995) of 5.1 to 5.4 seeds per capsule. This would firstly suggests the irrigation treatments did not affect the attractiveness of the flowers and effective pollinator activity was uniform across the site (House 1997). The small flowered eucalypt species such as *E. nites* are predominantly insect pollinated (Griffin and Ohmart 1986). Although the plantation was relatively exposed to the prevailing winds, it was a relatively warm site (Worledge *et al.* 1998) which promotes pollinating insect activity (Hingston and Potts 1998). The production rate of open-pollinated seed is therefore greatest under conditions of drought stress as trees in this environment produced the greatest number of mature capsules with no adverse effects on the number of seeds per capsule.

Lack of effective pollination may account for the reduction in number of seeds per capsule in the altitudinal transect as site altitude increased and umbel size decreased (Figure 2.9). The mean number of seed per capsule (*ca.* 1.8 to 2.7) was less than that found in the irrigation trial. Indeed, pollinator activity declines in the cooler conditions at higher altitudes (Savva *et al.* 1988). However, the numbers of seeds per capsule at all sites in the transect were still greater than those found in a coastal open pollinated seed orchard (*ca.* 1.7 to 1.8, Chapter 6) and may reflect higher rates of outcrossing encouraged by closer tree spacing (see review in Chapter 3). The effect of the number of capsules in an umbel in the altitudinal transect may be determined pre- or post-fertilisation. Larger umbels are more attractive to pollinators (House 1997) and Griffin (1980) found in *E. regnans*, that

the more flowers that were in an umbel, the longer the period in which the flowers opened. These pre-fertilisation factors may contribute to flowers in a larger umbel receiving disproportionately more pollen and setting more seed. However, the increase in seed numbers may be a response to post-fertilisation conditions where a larger umbel may also be a more competitive sink for assimilates (Lee 1988). Larger umbels may therefore be able to support the development of more seeds (Sedgley and Granger 1996) and at the same time maintain seed health. Indeed, the weight of the seeds is unaffected by the number of capsules in an umbel despite the increase in the number of seeds per capsule as umbel size increases (Figures 2.9 and 2.10).

Resource management and temperature during development appears to strongly control seed weight. In this experiment, seed weight was generally negatively correlated with the number of seeds per capsule and this was also found in *E. globulus* (Chapter 6). However at the 60 m site in the altitudinal transect this trend was reversed. This may reflect a higher rate of outcrossing as selfed seeds are generally smaller (Tibbits 1989) and better resource supply as there were more seeds per capsule creating a stronger resource sink (Lee 1988). A stronger resource sink may also account for the unchanging mean seed weight as umbel size increases despite the concomitant increase in seed number. Decreased pollinator activity as site elevation increases (Savva *et al.* 1988), may increase incidences of selfing and might also account for the concomitant decreases in seed weight. However, seed weight (Figure 2.10) may also be more directly affected by temperatures as this too decreased with increasing elevation (Table 2.4). However seed weight was not affected by maternal water availability in the irrigation trial despite the severe effects on growth and suggests a mechanism to protect the developing seeds. Bradford (1994) proposed a general model for developing seeds which included a semipermeable apoplastic membrane to maintain the optimum water potential around the developing embryo,

independent of the mother tree water potential. This model would appear to well represent the situation found in *E. nitens*.

The maternal tree from which the seed was harvested was one of the main factors affecting the number of seeds per capsule and seed weight across the sites in the altitudinal transect and in the irrigation trial (Tables 2.7 and 2.12). Similar significant maternal affects were also found in controlled pollinated *E. nitens* (Tibbits 1989) and open pollinated *E. globulus* (Hardner and Potts 1995). However, it is not clear if these effects are a reflection of the maternal genotype or microenvironment. These effects on seed weight would suggest that by screening bulk seedlots on the basis of seed weight to produce more uniform germination (Turnbull and Doran 1987), there would be disproportionate family representation in different weight fractions and this would narrow the genetic base of the planting stock (Barnett 1996). However, in *E. globulus* Martins-Corder *et al.* (1998) found no differences in genetic composition in bulk seedlot fractions classed by weight when tested using isozymes and suggest the practice is beneficial to nursery production and deployment. However this is yet to be validated for *E. nitens*.

Maternal environments which improve seed health impart a significant early establishment advantage on progeny as they benefit from both the success and rate of germination. Germination percentage and rate was highest in the irrigation trial and the lower altitude sites of the transect and (Figures 2.3, 2.4, 2.11 and 2.12). In the altitudinal transect, this corresponds to sites which had the greatest mean seed weight (Figure 2.10) whilst in the analysis of covariance, site effects were diminished when seed weight was accounted for though seed weight was not a significant influence (Tables 2.14 and 2.15). Because of their better nutrient and energy reserves, larger seeds of eucalypts have a greater advantage in nutrient poor conditions resulting in a higher percentage and rate of germination (Boden 1961, Turnbull and Doran 1987). Germination success and rate of *E. nitens* below the

optimum germination temperature (Donald and Jacobs 1993) is also benefited by higher seed quality. Seeds from the mild sites in the altitudinal transect had better germination percentage and rate at 12°C compared to seed origination from greater elevations (Figures 2.11 and 2.12). The effect of seed size has a significant affect on post emergent growth of eucalypts with seedlings from larger seeds exhibiting faster early rates of growth (Milberg and Lamont 1997, Milberg *et al.* 1998). It is not clear how persistent the effect of seed size is on seedling performance in *E. nitens* or if there are any other after effects of the maternal environment on the progeny as seen in many coniferous forest tree species (Stoehr *et al.* 1998) and this requires further investigation.

The maternal parent was a greater influence on seed germination success and rate than the maternal environment *per se*. The effect of the maternal parent on the seeds exhibited a degree of interaction to different germination temperature conditions (Table 2.15). Such interactions have been found in *E. delegatensis* and is suggested to represent an adaptive trait to different microclimates which may be experienced by germinating seeds (Battaglia 1997).

In summary, the quantity and quality of seeds produced by an *E. nitens* tree is significantly affected by the environment in which it grows. The effect of environment is expressed at a number of levels including the abundance of flowers per tree and their success in developing into seed bearing capsules, the number of seeds per capsule, the individual weight of seed, and the success and rate of germination. Maternal drought stress increased flower and capsule abundance and therefore seed production whilst flowering phenology, seeds per capsule, individual seed weight, germination success and rate are all unaffected. In contrast, increasing site altitude delays flowering and decreases the number of seeds per capsule, individual seed weight, germination success and germination rate. However, flower abundance was poorly related to site altitude and

appears to be influenced to some degree by site quality and frost. However, the maternal tree from which seed was harvested had the greatest effects on seed quantity and quality.

When selecting a site to establish an *E. nitens* seed orchard the environmental factors need to be favourable for both flower and seed production. There needs to be careful consideration of the climate, water and nutrient availability, the former the most critical as the other two factors can be ameliorated silviculturally. There is an obligate requirement of an extended period of low temperatures for flower induction in *E. nitens* (Moncur and Hasan 1994). However, achieving this by locating orchards at higher altitudes adversely affects seed quality. Sites which experience greater diurnal extremes of temperature would therefore appear to pose a favourable balance between the requirements of flower induction and seed quality.

The issue of water stress on flower abundance in eucalypts is a complex issue although the affects on phenology were not large. The results suggest productivity is enhanced by maintaining trees in a permanent state of water stress. However, consideration needs to be given to how this would affect the common practice in eucalypt orchards of paclobutrazol treatment. The most common mode of application of paclobutrazol is soil (collar) drench and under dry soil conditions, paclobutrazol binds to soil particles and is unavailable to the roots (Moncur *et al.* 1994b). Therefore if intending to treat with paclobutrazol, it would be better to ensure an adequate supply of water to the trees.

There is strong evidence in many species that the maternal environment can affect progeny for many years after germination (Roach and Wulff 1987). This is yet to be explored in eucalypts and may change the way in which seed orchards are managed in the future.

Chapter 3

The effect of tree spacing on the production of flowers and capsules

3.1 INTRODUCTION

In *Eucalyptus nitens*, the means of mass producing seeds of high genetic quality has focused on maximising yield from individual trees rather than whole orchard output (see Moncur 1998). This is possibly a consequence of the complexity and commitment required to study, let alone manipulate, whole orchard systems. Many current eucalypt seed orchards were initially progeny trials which have been culled (Moncur 1998). Orchards such as these may have average spacings of 10 metres or more between trees. Wide spacings with better access to light and low competition for resources promotes good crown development and opportunities for flower development (Moncur 1998). However in this system, the crowns are several metres off the ground and ladders or hoists are required to reach them for capsule harvesting (Swain and Chiappero 1998). Management, infrastructure and maintenance costs could also be expected to be higher with trees spread over a broad area (Bouvet 1997).

There are concerns about the quality of seeds derived from widely spaced trees. Although seed orchards do offer higher rates of outcrossing than in natural stands (Moran *et al.* 1989), there was a noticeable reduction in viability of seeds harvested from an *E. grandis* seed orchard with a 8.2 x 8.2 m spacing compared to seed harvested from a more closely

spaced plantation (van Wyk 1981). *Eucalyptus nitens* is self fertile to some degree (Tibbits 1989) and there is significant inbreeding depression in the major growth traits (Hardner and Tibbits 1998). It is not possible to cull inbred seedlings in the nursery as inbreeding depression in eucalypts usually does not become apparent until after seedling outplanting (Potts and Wiltshire 1997). Eucalypts have a mixed mating system and whilst protandry offers some barrier to self pollination within a flower (autogamous pollination), pollination from other flowers on the same tree (geitonogamous pollination) occurs readily (Pryor 1976).

The small cream coloured flowers of *E. nitens* which generally begin to open in early summer lend themselves to pollination by insect vectors (Griffin and Ohmart 1986, House 1997). One potential pollinator which has been promoted to benefit seed production in eucalypt orchards is the honey bee (*Apis mellifera*). When bees were introduced to *E. globulus* orchards, the number of seeds per capsule was increased whilst in *E. nitens* orchards, outcrossing rates were increased with the introduction of bees (Moncur *et al.* 1995). However, bees have a characteristic foraging pattern which under certain circumstances may enhance geitonogamous pollination. Studies in almond orchards on the pollen collected by bees on returning to the hive revealed little or no genetic diversity in each pollen load and indicated bees on each flight foraged exclusively on one tree or at most one cultivar (Jackson and Clarke 1991).

Other effective insect vectors of small flowered eucalypts include flies and wasps (Griffin and Ohmart 1986). Strong flying insects could potentially carry pollen for days over long distances (Ashton & Sandiford 1988). However, like bees, a number of insect species have been observed to restrict foraging to flowers of a single canopy, potentially mediating geitonogamous pollination (Griffin 1980, Hingston and Potts 1998). The actual pollen

dispersal pattern from the original tree is most likely highly leptokurtic (Barber 1965). The most commonly quoted figure for the mean effective pollination distance in an *E. regnans* orchard is that of 42 m found by T. Adams (Sedgley and Griffin 1989, Moncur and Kleinschmidt 1992, House 1997). However, in an insect-pollinated tropical dioecious tree species, pollination rates fell to less than half when the distance between female and nearest male was only 6.5 m (House 1993). In *Mimulus ringens*, an insect-pollinated perennial with a mixed mating system, both the frequency of inter-plant movement of pollen vectors and rates of outcrossing increased as population density increased (Karron *et al.* 1995).

It is not only tree density which appears to moderate pollen vector movement. Trees which are able to attract a greater number of pollen vectors appear to have greater reproductive success (House 1997). However, geitonogamy appears to increase with increasing numbers of open flowers on a plant (de Jong *et al.* 1993). Indeed, a lower number of flowers per tree may promote more inter tree movement of vectors and therefore greater levels of outcrossing (House 1997).

It appears that to optimise the productivity in both quantity and genetic quality of seed produced from an *E. nitens* seed orchard, two main opposing factors must be balanced. Firstly, is the need for sufficient space between trees to promote canopy development and the living framework to produce and carry large seed crops. Opposing this is the need to locate trees closely to encourage as many inter-tree movements of pollen vectors as possible in order to maximise outcrossing and minimise geitonogamy. As seed production moves from orchards derived from progeny trials to clonal seed orchrds the need to rogue will be greatly diminished as much of the selection procees for desirable traits is carried out before planting. This importaince of selecting the optimum spacing at planting will

become more crucial. In this chapter, the first step in characterising the relationship between reproductive density in *E. nitens* has been undertaken. The effect of tree spacing on the production of flowers and capsules on a per tree and per hectare basis was studied in two trials. The results and directions for further research and implications on commercial production are discussed.

3.2 METHODS AND MATERIALS

3.2.1 Study sites

Two pre-established *Eucalyptus nitens* spacing trials were used in this study. The first was established in 1992 by Forestry Tasmania (FT) at a site near Castra 15 km south of Ulverstone in northern Tasmania. The second site was established in 1984 by Australian Pulp and Paper Mills Ltd. (now North Forest Products [NFP]) at a site known as Ringwood, 34 km south of Burnie in north-western Tasmania.

The Castra trial was established on a fertile, ex-pasture site which receives a mean annual rainfall of 1250 mm and is situated at 310 masl. Planting took place in late winter 1992 where 24 experimental plots were established, each 30 x 30 m at one of six different spacings replicated four times in complete randomised blocks. In the centre of each experimental plot was a 25 tree measurement plot (Neilsen and Gerrand 1999).

The Ringwood trial was established in an area previously covered with eucalypt-myrtle forest which receives a mean annual rainfall of 1800 mm and is situated at 550 masl

(Wang *et al.* 1998). The site was planted in early summer 1984 in a modified Nelder design which consisted of 35 radiating spokes separated by 3 degrees at their origin. Each spoke was intersected by 24 concentric arcs. Each arc was spaced such that the distance between each arc as they intersected a spoke was the same distance separating the spokes in that arc. This design produced a constant rectangularity as the spacing increased with increasing distance from the origin of the spokes and inter-arc spacings ranged from 1.5 to 5.0 m. (Fisk and Docking 1984).

Both sites were intensively managed before and after planting to suppress weed competition and predation. Any deaths up to six months of age were replaced with surplus stock of the same origin (Neilsen and Gerrand 1999, Fisk and Docking 1984).

3.2.2 Flowering and capsule survey

In mid-November 1997 litter traps were established in both the Castra and Ringwood spacing trials, approximately 5 and 13 years after planting respectively. In the Castra trial, one trap was randomly placed in the measurement plot of five of the six spacings in each of the four replicates (Table 3.1) for a total of 20 traps. In the Ringwood trial, five traps were placed randomly between spokes within arcs which correspond to average spacings of 1.54, 2.00, 3.04, 3.70 and 4.62 m between trees (Table 3.1) for a total of 25 traps.

The litter traps were constructed from Weathashade® 70% shade cloth suspended at each corner from 80 cm galvanised steel posts. Each trap was of a size and shape such that each of the four corners rested against the trunk of a tree, thus the area sampled was equivalent to the spacing of each tree. The canopy sampled was equal to one quarter that

of each of the four trees surrounding the trap and therefore considered equivalent to one whole tree. The centre of each trap was secured by a steel wire to a pin embedded in the ground, this formed the trap into a shallow cone and prevented the contents of the trap being blown out.

Table 3.1 Tree spacings and respective planting densities of the Forestry Tasmania (FT) Castra and North Forest Products (NFP) Ringwood trials examined for flower and capsule production.

Trial	Spacing between trees (m)	Planting density (stems per hectare)
Castra (FT)	3.00 x 2.00	1667
	3.00 x 2.50	1333
	3.30 x 3.00	1010
	4.00 x 3.00	833
	5.00 x 4.00	500
Ringwood (NFP)	1.54 x 1.54	4216
	2.00 x 2.00	2500
	3.04 x 3.04	1082
	3.70 x 3.70	730
	4.62 x 4.62	468

The litter in each trap was collected at intervals of *ca.* 7 weeks until the final collection in mid-November 1988. Any bare branches longer than 1 m found in the traps were not collected. The litter was air dried then weighed prior to hand sorting. In each collection the numbers of (i) buds still enclosed in bracts, (ii) unopened flower buds after bract shed, (iii) opercula, (iv) opened flower buds/immature capsules (valves still closed) and (v) mature capsules (valves opened) were counted. The annual totals for each trap for the reproductive structures and litter weight was calculated prior to analysis.

3.2.3 Trial measurement

In May 1997 (4.5 years after planting) the diameter at breast height over bark (DBH) of all trees in the measurement plots of the Castra trial were measured and plot means were kindly supplied by W. Neilsen of Forestry Tasmania. In December 1998 (14 years after planting) all trees in the NFP trial were measured for DBH.

3.2.4 Statistical analysis

From the annual totals for each reproductive structure and litter weight for each trap values was calculated for (i) the annual totals per hectare based on the area covered by each trap and (ii) the numbers of each reproductive structure per kg of litter. A statistical model was then fitted for each reproductive structure and litter weight on a per trap (and therefore per tree), per hectare and per kg of litter basis and for the proportion of flowers which opened but were lost before reaching maturity. The model fitted to the data from the Castra trial was:

trait = mean + rep + spacing + error.....(Eqn. 3.1),

whilst the model fitted to the data from the Ringwood trial was:

trait = mean + spacing + error.....(Eqn. 3.2),

where in both Eqn. 3.1 and 3.2, spacing is the fixed effect of tree spacing whilst in the Castra trial (Eqn 3.1), rep is the random effect of the litter trap replicate. Analysis was also undertaken to examine and remove the effect of tree size by including mean DBH as a covariate in Eqn. 3.1 and 3.2. For the Castra trial, plot means for DBH were used whilst for the Ringwood trial, mean DBH for the trees at each corner of the trap were used. These measurements were also used to analyse the spacing effect on DBH alone. In each analysis the data was transformed as necessary to optimise the normality of the residuals and homogeneity of the variances then back transformed for presentation on a per tree and per hectare basis. For each trait, specific *a priori* contrasts between the spacings were undertaken using Tukeys adjustment and least squares means and standard errors of the spacing levels calculated. The statistical model was initially fitted with the PROC GLM procedure in SAS (SAS 1992) to examine the need to transform the raw data. The model was then fitted again to the data (which was transformed as necessary) using the PROC MIXED procedure in SAS (SAS 1992) to generate the specific contrasts, least squares means and standard errors. Pearsons correlations between both the number of buds enclosed in bracts and the number of mature capsules with the number of opercula were carried out using the PROC CORR procedure in SAS (SAS 1992). The data from the fifth trap in the 3.70 x 3.70 m spacing at the Ringwood trial were excluded from the analysis as there were few (< 8) or no reproductive structures found over the whole year. This trap alone was located in an area of the trial prone to waterlogging which may have contributed to the reduced flower production.

3.3 RESULTS

Most of the litter fall including flower parts occurred in the summer and autumn months at both Castra and Ringwood (Figure 3.1). However, the fall of mature capsules occurred with reasonable uniformity through out the year at Ringwood but increased late in the year at Castra (Figure 3.1).

The spacing between trees significantly affected growth and the number of opercula, capsules and leaf litter on a per tree basis at both the 5 year old Castra site ($p < 0.05$) and the 13 year old Ringwood site ($p < 0.001$, Table 3.2). More specifically, at both sites, there were significant effects on- the number of flowers which opened, the number of buds with opercula which were lost, the number of opened flowers and immature capsules which were lost, the mature capsule fall and the total mass of litter fall on a per tree basis. Tree DBH was also significantly affected by spacing (Table 3.2). At the Ringwood site only, spacing also significantly affected the number of buds in bracts which were lost and the proportion of opened flowers which were lost on a per tree basis (Table 3.2).

The general effect of increasing spacing between trees at both sites was an increase in tree DBH, the number of flowers which opened, the number of reproductive structures which were lost and the mass of litter on a per tree basis (Figures 3.2 to 3.7 and 3.9). At Ringwood, the proportion of opened flowers which aborted was significantly affected by spacing, with the rate significantly reduced in the most extreme close spacing (Figure 3.7). The number of opercula was significantly positively correlated with both the number of buds in bracts (Ringwood $r = 0.82$, $p < 0.001$; Castra $r = 0.62$, $p < 0.01$) and the number of mature capsules (Ringwood $r = 0.86$, $p < 0.001$; Castra $r = 0.55$, $p < 0.05$).

Tree size could not account for the significant effect of spacing on the number of flowers which opened, litter produced and reproductive structures lost per tree at the older Ringwood site. The effect of spacing on these traits remained significant at a per tree level when DBH was fitted as a covariate, with the exception of the number of buds in bracts lost (Table 3.3). This was in contrast to the younger Castra site where the spacing effects on flowering and the loss of reproductive structures were due to tree size and became insignificant when DBH was fitted as a covariate, the only exceptions in this case being the significant effect of spacing on mature capsule fall and litter weight which remained significant (Table 3.3).

On a per hectare basis, the effect of spacing between trees significantly affected the number of flowers which opened, the number of buds in bracts and buds with opercula which were lost and, the total mass of litter collected at the Ringwood site (Table 3.2). These traits tended to increase per hectare as the spacing between trees increased as seen on the per tree basis (Figures 3.2 to 3.4 and 3.7) However, spacing had no significant effect on reproductive output and litter fall on a per hectare basis at the younger Castra site except for the number of buds in bracts which were lost (Table 3.2).

There was very little difference between the sites in both the amount of litter produced per hectare (Figure 3.7) and the proportion of open flowers which aborted (Figure 3.8), despite the differences in age and diameter between the sites (Figure 3.9). However, the older Ringwood trial produced substantially more flowers (approximately 4 fold) than the Castra trial at similar planting densities (Figure 3.4). Despite this, there was no significant difference between the spacings in the number of any reproductive structure on a per kg of litter basis at either site (Table 3.2).

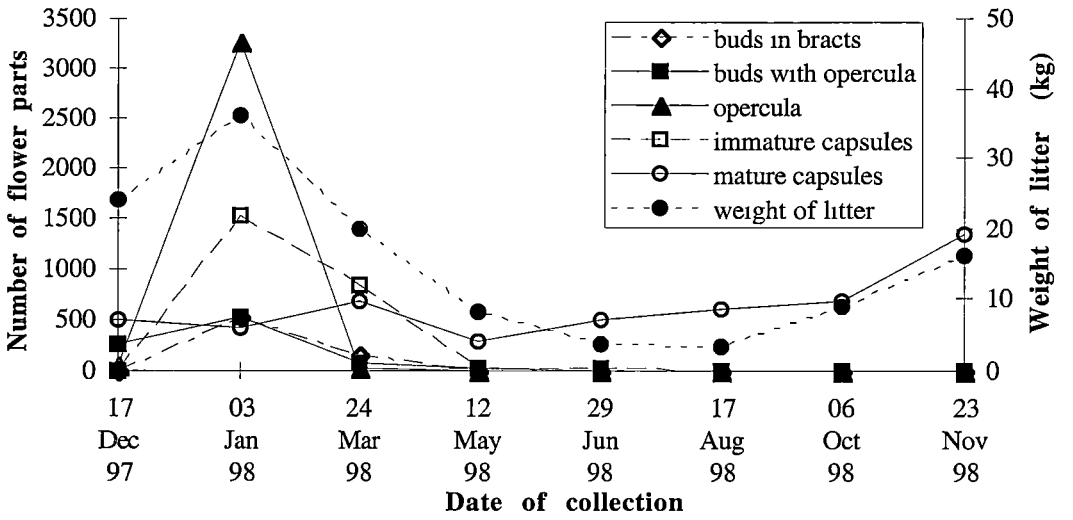
Table 3.2 F values from ANOVA for the effect of spacing on the quantity of reproductive structures, litter and growth for the spacing trials at Castra (5 years old, DF = 4, Error DF = 12) and Ringwood (13 years old, DF = 4 Error DF = 19). Traits were analysed on a per tree, per ha and per kg of litter basis. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, .= not analysed.

	Castra			**Ringwood**		
	Per tree	Per ha	Per kg of litter	Per tree	Per ha	Per kg of litter
Buds in bracts	2.78 n.s.	5.69 **	0.80 n.s.	3.87 *	2.92 *	0.82 n.s.
Buds with opercula	3.95 *	1.45 n.s.	1.26 n.s.	9.65 ***	5.40 **	1.83 n.s.
Opercula	4.15 *	2.24 n.s.	2.19 n.s.	21.72 ***	6.06 **	2.71 n.s.
Open flowers and immature capsules	4.58 *	2.17 n.s.	2.16 n.s.	21.65 ***	2.37 n.s.	2.50 n.s.
Proportion of open flowers which aborted	0.11 n.s.	. .	. .	5.00 **	. .	. .
Mature capsules	3.83 *	2.09 n.s.	1.93 n.s.	21.18 ***	2.61 n.s.	2.45 n.s.
Weight of litter	24.36 ***	1.51 n.s.	. .	331.61 ***	13.21 ***	. .
Diameter at breast height over bark	4.06 *	. .	. .	14.93 ***	. .	. .

Table 3.3 F values from ANOVA for the effect of tree spacing on the quantity of reproductive structures and litter on a per tree basis with mean tree diameter (DBH) as a covariate for the spacing trials at Castra (5-6 years old) and Ringwood (13-14 years old). Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

	Castra		**Ringwood**	
Effect	*covariate (DBH)*	*Spacing*	*covariate (DBH)*	*Spacing*
DF	1	4	1	4
Error DF	11	11	18	18
Buds in bracts	1.61 n.s.	2.10 n.s.	0.09 n.s.	1.06 n.s.
Buds with opercula	0.20 n.s.	2.81 n.s.	0.00 n.s.	3.13 *
Opercula	1.54 n.s.	2.43 n.s.	0.15 n.s.	6.88 **
Open flowers and immature capsules	1.56 n.s.	2.81 n.s.	0.36 n.s.	7.83 ***
Proportion of open flowers which aborted	0.38 n.s.	0.18 n.s.	0.17 n.s.	3.36 *
Mature capsules	0.91 n.s.	3.85 *	0.60 n.s.	7.36 **
Weight of litter	0.04 n.s.	13.18 ***	3.76 n.s.	122.61 ***

(a) Castra



(b) Ringwood

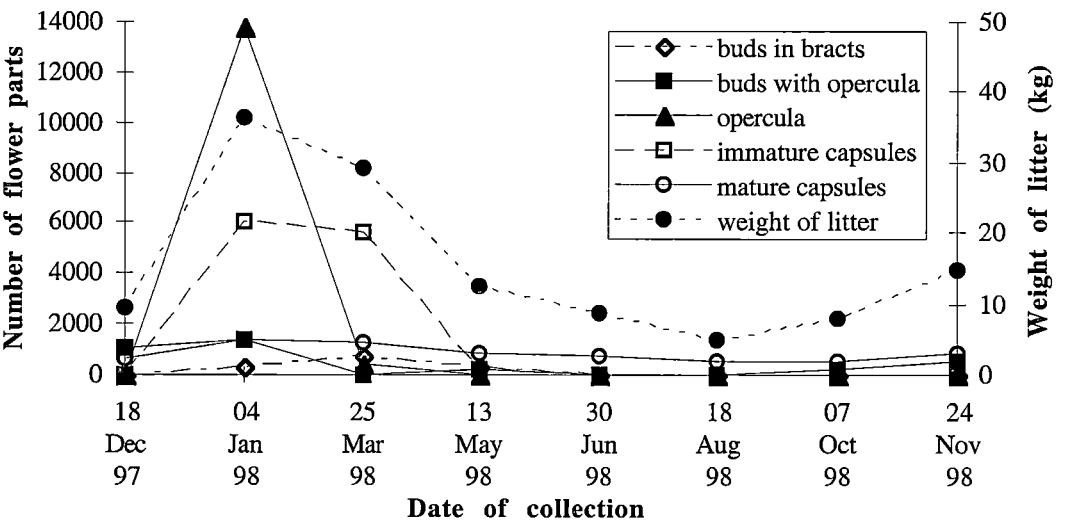
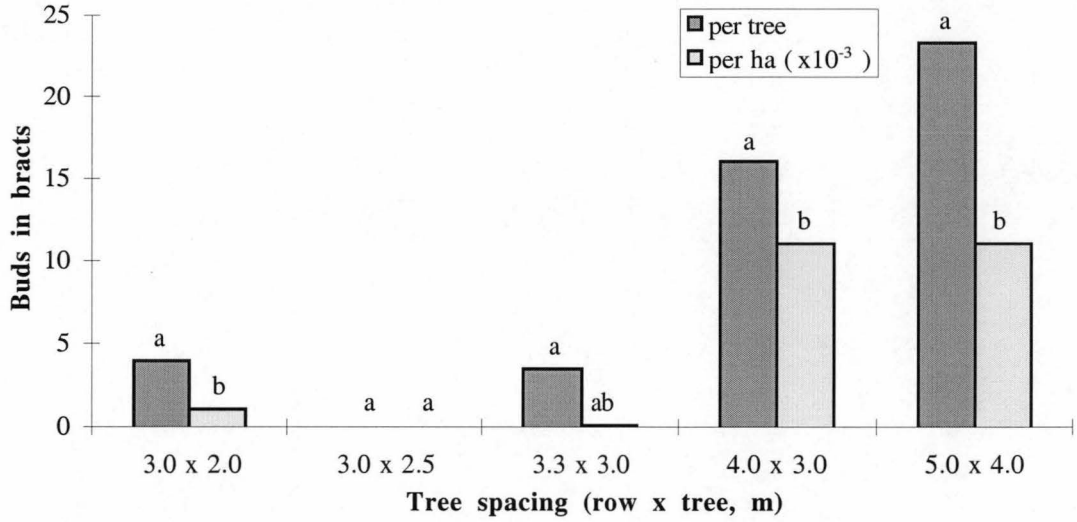


Figure 3.1 The total number of *E. nitens* flower parts and weight of litter from all litter traps in each collection made over a 1 year period from the spacing trials at (a) Castra and (b) Ringwood. Litter collection commenced on the 17th of November 1997 at Castra and the 18th of November 1997 at Ringwood.

(a) Castra



(b) Ringwood

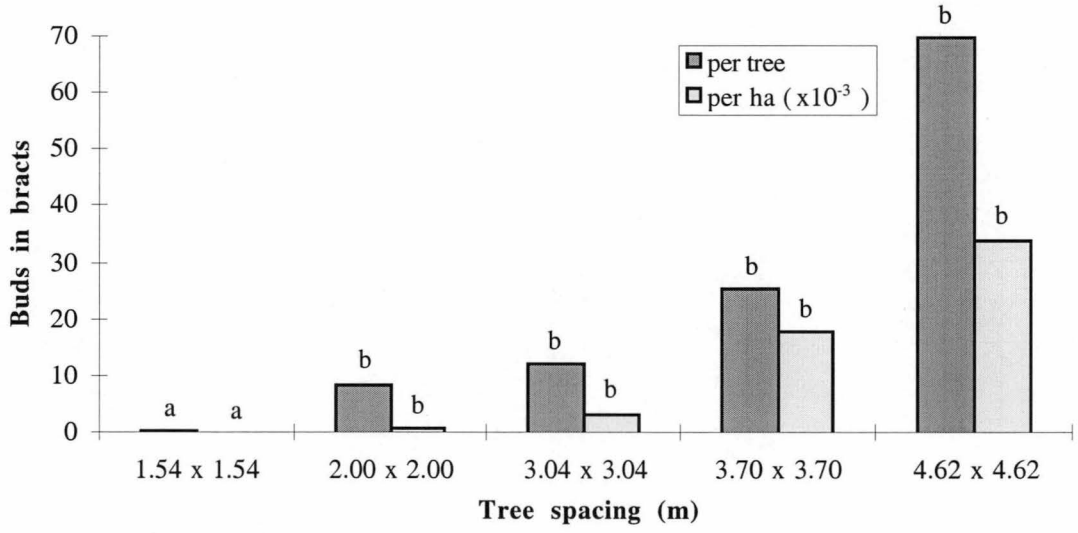
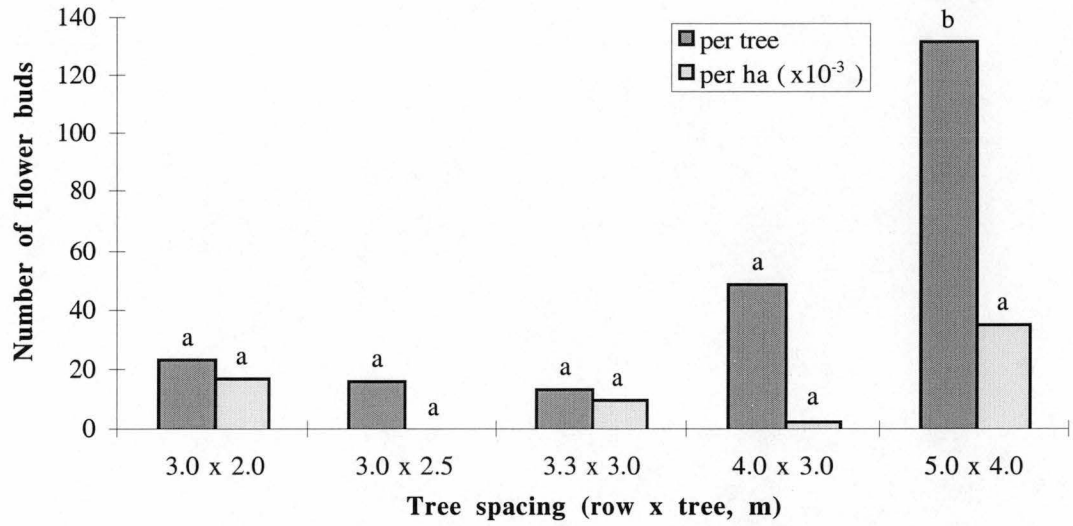


Figure 3.2 The mean number of *E. nitens* flower buds enclosed in bracts lost at different tree spacings in the (a) Castra and (b) Ringwood trials. Values in the same series with the same letter are not significantly different ($p > 0.05$) based on Tukeys test for multiple comparisons.

(a) Castra



(b) Ringwood

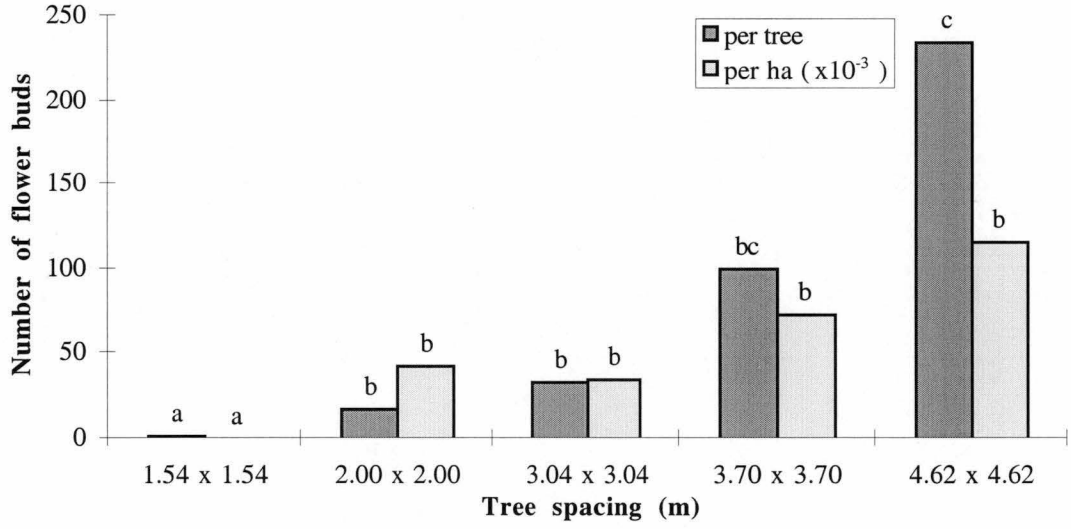
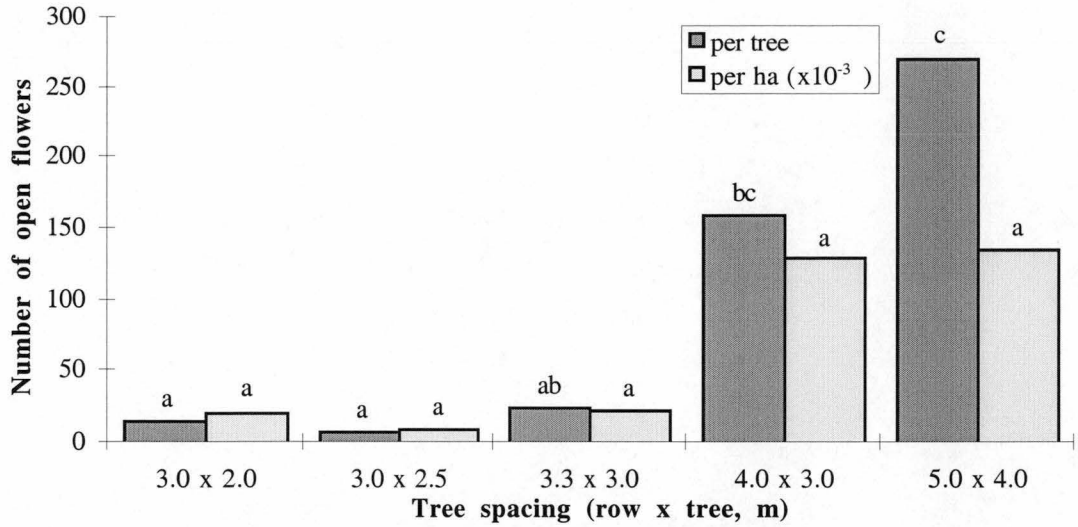


Figure 3.3 The mean number of *E. nitens* flower buds which were lost before opening, collected at different tree spacings in the (a) Castra and (b) Ringwood trials. Values in the same series with the same letter are not significantly different ($p > 0.05$) based on Tukeys test for multiple comparisons.

(a) Castra



(b) Ringwood

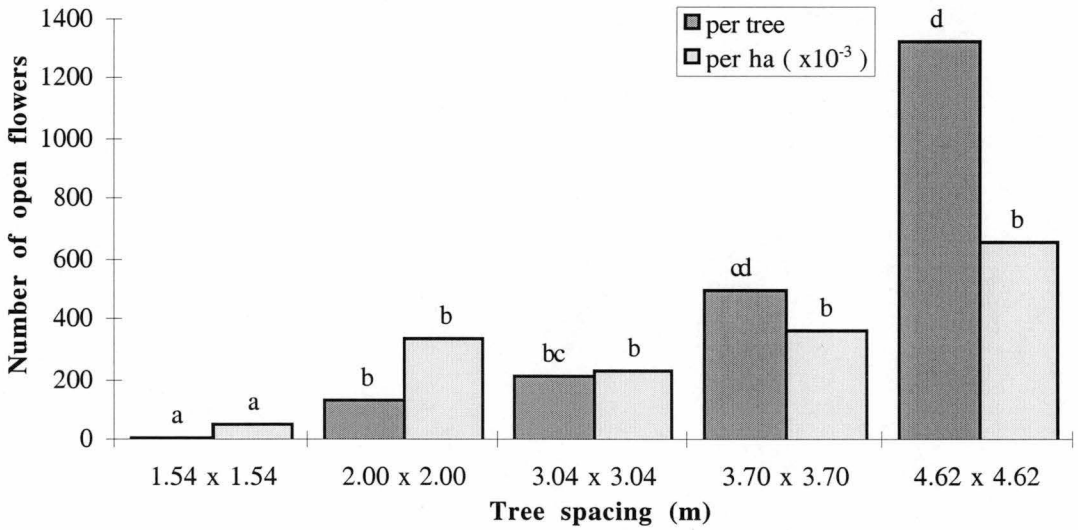
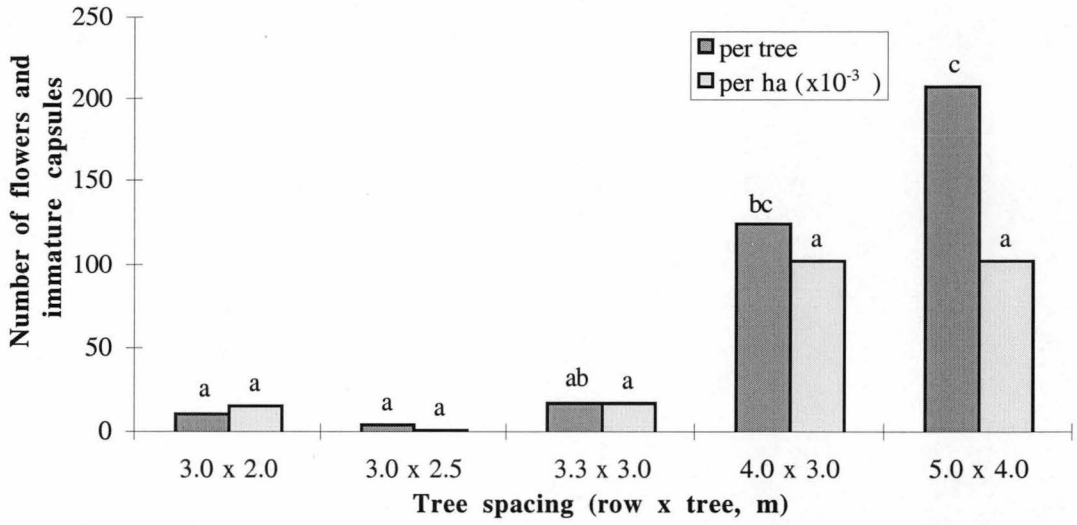


Figure 3.4 The mean number of *E. nitens* flowers which opened (based on opercula collected in litter traps) at different tree spacings in the (a) Castra and (b) Ringwood trials. Values in the same series with the same letter are not significantly different ($p > 0.05$) based on Tukeys test for multiple comparisons.

(a) Castra



(b) Ringwood

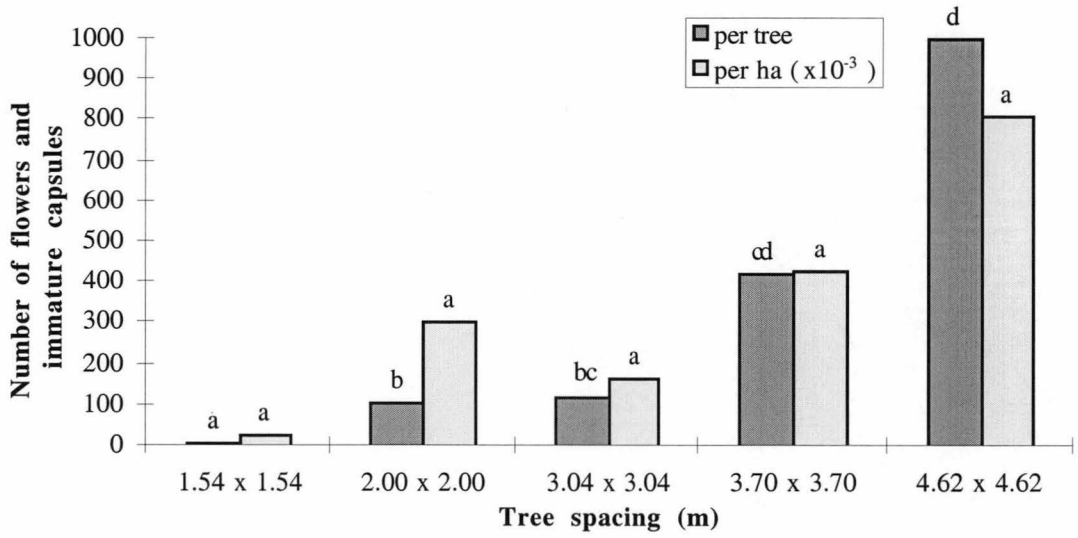
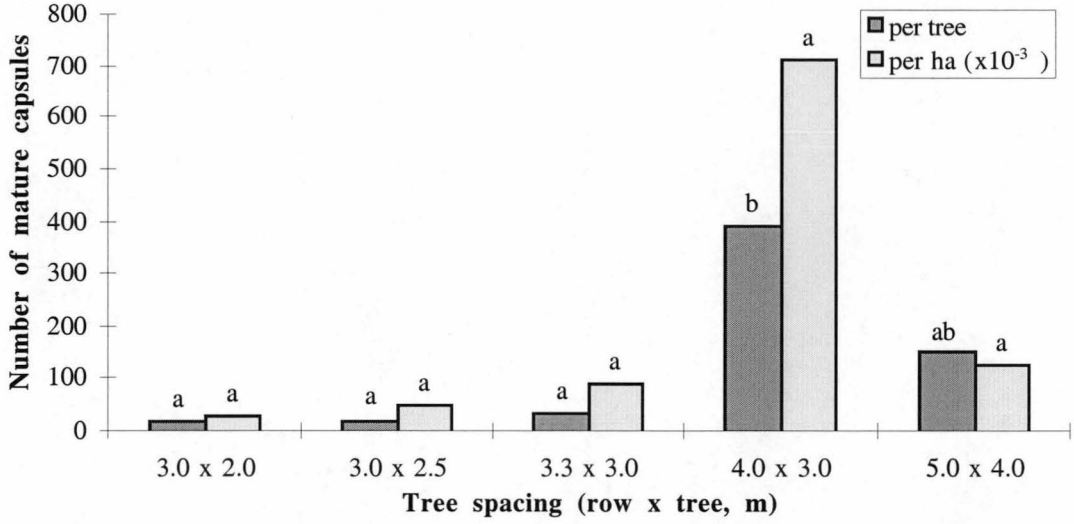


Figure 3.5 The mean number of open *E. nitens* flowers and immature capsules which were lost at different tree spacings in the (a) Castra and (b) Ringwood trials. Values in the same series with the same letter are not significantly different ($p > 0.05$) based on Tukeys test for multiple comparisons.

(a) Castra



(b) Ringwood

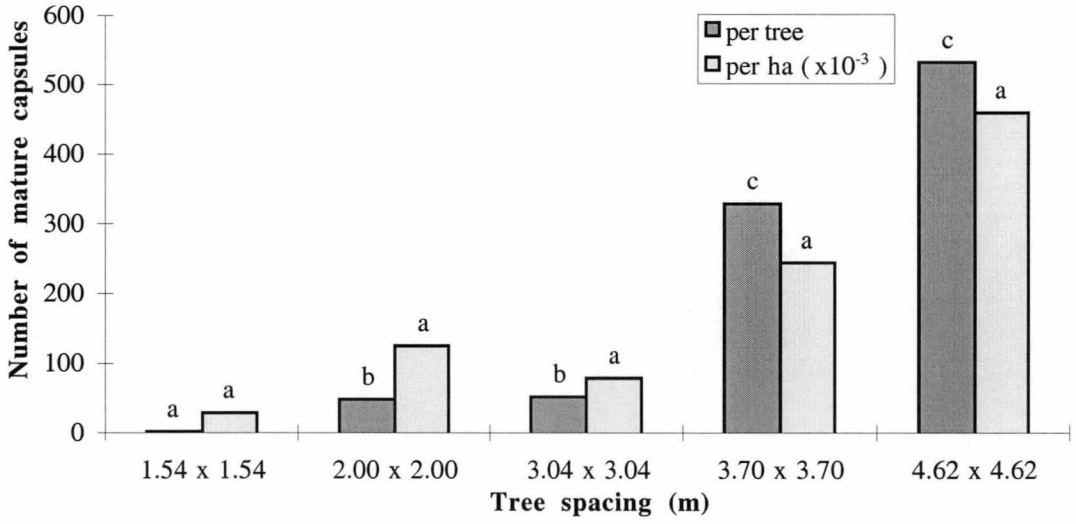
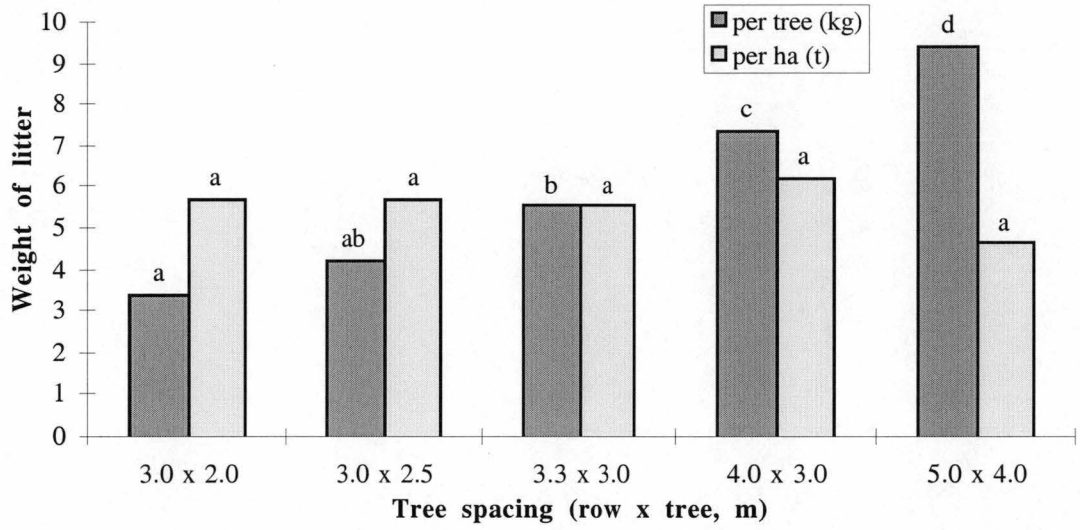


Figure 3.6 The mean number of mature *E. nitens* capsules of undetermined age collected in litter traps at different tree spacings in the (a) Castra and (b) Ringwood trials. Values in the same series with the same letter are not significantly different ($p > 0.05$) based on Tukeys test for multiple comparisons.

(a) Castra



(b) Ringwood

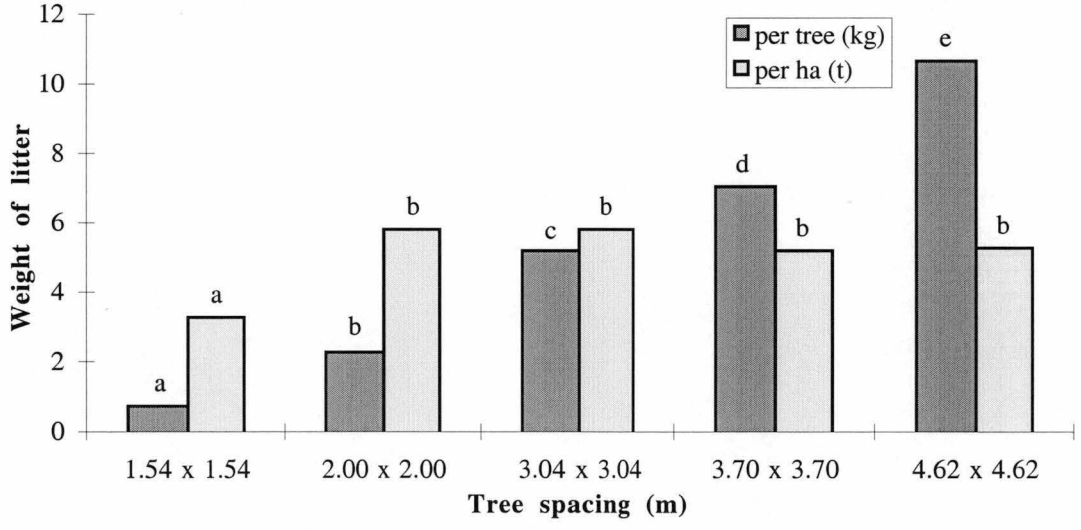
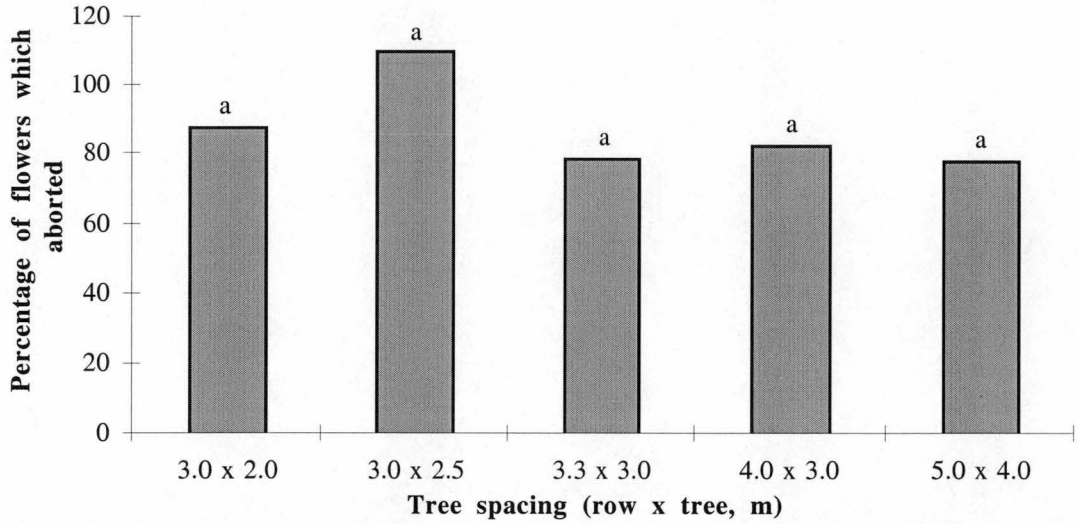


Figure 3.7 The mean weight of *E. nitens* leaf litter (kg or t) collected in 12 months at different spacings in the (a) Castra and (b) Ringwood trials. Values in the same series with the same letter are not significantly different ($p > 0.05$) based on Tukeys test for multiple comparisons.

(a) Castra



(b) Ringwood

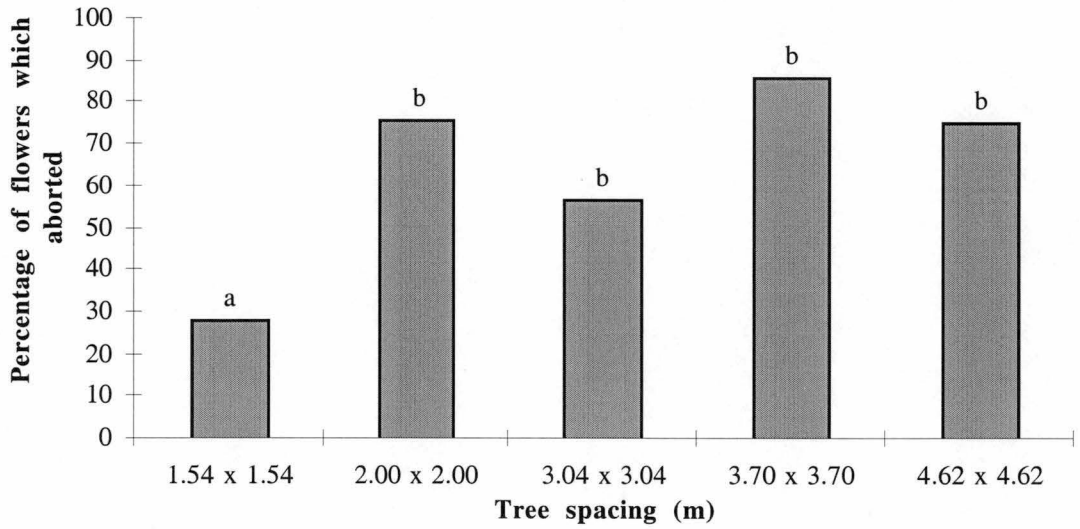


Figure 3.8 The percentage of *E. nitens* flowers which opened but were lost before maturing into capsules at different tree spacings in (a) Castra and (b) Ringwood trials. Values with the same letter are not significantly different ($p > 0.05$) based on Tukeys test for multiple comparisons.

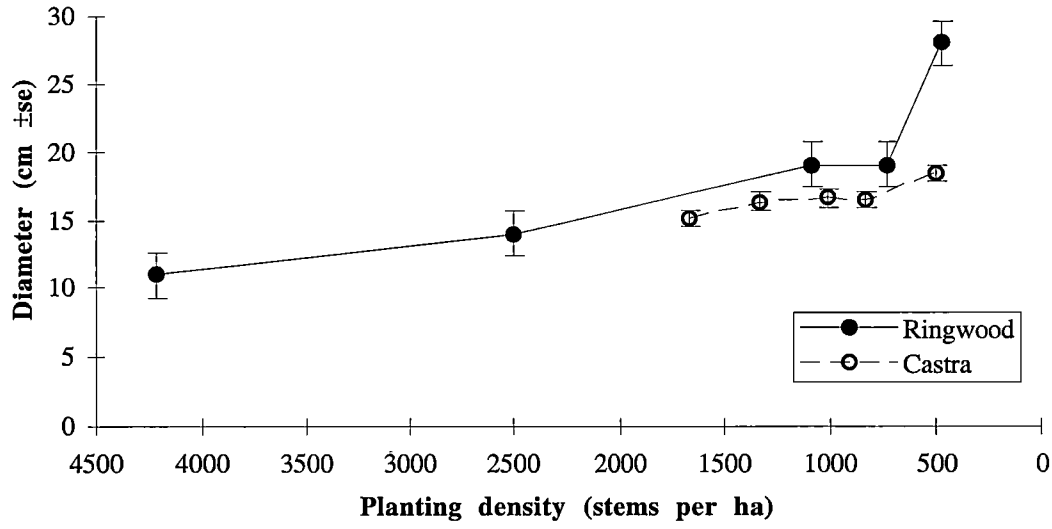


Figure 3.9 The diameter at breast height of *E. nitens* trees at different planting densities in 2 separate trials. Trees at Ringwood were measured in December 1998 when 14 years old whilst trees in the Castra trial were measured in May 1997 when 5 years old.

3.4 DISCUSSION

Tree spacing had a significant effect on the number of reproductive structures collected in litter traps on a per tree basis at the Ringwood site and a lesser effect at the Castra site (Table 3.2) which was younger and had a narrower range of spacing classes represented (Figure 3.9). As spacing increased so too generally did the number of flowers which opened, the number of reproductive structures shed and the weight of litter fall for each tree. While this general trend persisted, the significance of the effect of spacing was diminished when these traits were expressed on a per hectare basis (Table 3.2). Nevertheless, at the Ringwood site there was a clear and significant increase in the number of flowers produced per hectare as spacing between trees increased. Individual tree DBH was also significantly affected by spacing (Table 3.2). When DBH was fitted as a

covariate to the effect of spacing on the reproductive and litter traits, the significance of the spacing effect was diminished at both sites (Table 3.3). Despite this, the effect of spacing on reproductive traits at the Ringwood site was still significant and therefore could not be wholly attributable to tree size (Table 3.3).

The effect of spacing on flower production in the present case is likely to be stable across years. The size of the present, and immediately preceding and subsequent flower crops could be estimated by the number of opercula, mature capsules and buds in bracts which were collected respectively. In each case there was a general trend with an increase in tree spacing to increase the size of the flower crop, not only on an individual tree basis but also on a per hectare basis at both the Castra and Ringwood sites (Figures 3.2, 3.4 and 3.6). Indeed, both the number of buds in bracts and mature capsules collected were strongly correlated with the number of opercula at both sites. The estimate for the number of flowers which opened is a particularly robust figure as each operculum collected is directly equivalent to an open flower in the canopy. This suggests the results for the number of buds in bracts and mature capsules collected is indicative of the numbers in the canopy and the effect of spacing on these crops of the past and future is as significant as in the present crop.

It is not expected that results from either the Ringwood or Castra trials were influenced inequitably by the macroenvironment. In *E. nitens*, flowering success appears to be related to an obligate requirement for a period of cold (Moncur and Hasan 1994) and the presence or absence of good flowering years has been attributed to cold or mild winters respectively (Eldridge and Griffin 1990, Swain and Chiappero 1998). As the sites covered only a few hectares of flat ground, differences in temperature across the sites would be expected to be uniform and not have a significant affect on the results. Ashton

(1975) found a close relationship between bad flowering years in *E. regnans* and years of low rainfall. However, there was no significant difference in the number of flowers produced by equally spaced *E. nitens* trees that were well watered compared to trees under continuous water stress (Chapter 2). This suggests that if there was any level of water stress in the trials it would not have affected flowering.

In the Castra trial, there was a degree of competition observed at all spacings indicated by the lifting of the green crown (Neilsen and Gerrand 1999) and this was also observed at the Ringwood trial. This would reduce the potential flower producing capacity thus the figures for flowers per tree at the widest spacing do not represent the maximum production per tree possible at either site. The strong positive trend observed in the numbers of flowers per tree with increasing spacing at both Castra and Ringwood and per hectare at Ringwood (Figure 3.4) could therefore be expected to persist at a wider spacing. However, it is not clear from these results what the optimum spacing might be for these trials though it does appear to increase as trees mature.

There is a risk of decreasing outcrossing and increased geitonogamy in going to a wide spacing (Karron *et al.* 1995), the relative size of this risk is yet to be determined in *E. nitens*. Indeed, there may be an advantage to limiting the numbers of flowers per tree whilst maintaining the maximum flowers per hectare. As the number of open flowers per tree increase so to does the rate of geitonogamy (de Jong *et al.* 1993). Shea (1987) found in Engelmann spruce that the levels of outcrossing were higher in medium sized trees than in larger trees and it was believed that larger trees had more male cones thus in the immediate environment of the tree, the density of its own pollen was greater.

While the crowns contacted in all spacing treatments studied, the degree of contact increased with increasing stocking rate. Crown contact is thought to encourage insect movement between canopies (van Wyk 1981, Jackson and Clarke 1991, Eldridge *et al.* 1993, Jackson 1996). However, it is not clear if this resulted in the relatively uniform rate of flower abortion across the spacings (Figure 3.8), with only the extreme close spacing in the Ringwood trial (1.52 x 1.54 m) significantly lower. It is common that eucalypts abort a high percentage of opened flowers (Moncur *et al.* 1992). The relative rate of flower abortion found here of approximately 70 to 80 % was slightly higher than that of an intensively managed coastal seed orchard (5 x 5 m spacing) where approximately 60% of flowers which opened did not mature into capsules (Chapter 6).

The results from the Castra trial would suggest that on a per hectare basis, planting densities of 833 to 494 stem per hectare would be close to optimal for flower and capsule production in the early reproductive life of the orchard. However, the results from the Ringwood trial suggest that as trees become larger and reproductively fully matured, density may need to be decreased to maintain productivity. However, the application of growth retardants such as paclobutrazol may negate the need for thinning to increase spacing between trees. Currently there are *E. nitens* clonal seed orchards planted or being planted in Tasmania at densities of *ca* 400 stems per hectare. These have been or will be treated with paclobutrazol and will provide an opportunity to examine the effectiveness of this management regime on seed production.

Close spacing of trees would improve land utilisation and reduce the general management costs (Bouvet 1997). However, there is a risk that too small a spacing between trees (spacings < 5 m or > 400 stems per hectare) may make it difficult to bring harvesting equipment, such as hoists, into the orchard (Swain and Chiappero 1998). Careful orchard

planning might satisfy a high density arrangement for promoting effective inter-tree movement of pollen vectors as well as having sufficient space for ease of harvesting. Eldridge *et al.* (1993) suggest a hedge row design with trees closely spaced within rows to encourage extensive vector movement and wide inter-rows for access with machinery. In the present results, it is not clear what the effects of a hedgerow arrangement might have on tree survival and flower and capsule production and this would require further investigation.

In summary, it has been shown that planting density significantly affects the production of flowers and capsules on both a per tree and per hectare basis. Furthermore, the relative degree to which spacing affects these traits appears to increase as trees mature. The focus of interest now needs to be moved onto seed production. There are three main aspects of seed production that would need to be addressed that may be affected by spacing. Firstly, are the pollinator activities and outcrossing rates affected? Secondly, are the numbers of seeds per capsule and per hectare affected? Finally, is the physiological quality of the seed affected?

Chapter 4

Response of *Eucalyptus nitens* seedlings to gibberellin biosynthesis inhibitors

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4.1 INTRODUCTION

One of the most significant developments in eucalypt seed production was the recognition that the gibberellin (GA) biosynthesis inhibitor, paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-1,2,4-triazol-1-yl-pentan-3-ol or PP333], was capable of inducing precocious and abundant flowering without degrading seed quality. Paclobutrazol, a triazole derivative, inhibits the GA biosynthesis pathway between *ent*-kaurene and *ent*-kaurenoic acid (Graebe 1987). Initially tested in *E. globulus* to control vegetative growth (Hetherington and Jones 1990), paclobutrazol was later found to enhance flowering in mature *E. globulus* and *E. nitens* trees (Griffin *et al.* 1993) and induce *E. globulus* seedlings to flower at less that two years of age, three years ahead of their normal reproductive development (Hasan and Reid 1995). The ability of paclobutrazol to inhibit the synthesis of GAs in *E. nitens* was identified as a key factor in

its effectiveness in enhancing flowering, with a strong relationship being found between reduced levels of GA_1 and increased flowering abundance (Moncur and Hasan 1994).

Paclobutrazol can persist in the soil for several years as it is generally resistant to chemical and biotic degradation (Jackson *et al.* 1996) and mass movement (Leaver 1986). The impact of a high dose of paclobutrazol on *E. globulus* trees has been detected up to 69 months after treatment (Griffin *et al.* 1993). The long term effects on plants of a chemical with such a high residual activity can be difficult to regulate. There would be a high risk of over supply and the potential for deleterious inhibition of growth and development (Curry and Reed 1989).

There are a number of alternative GA biosynthesis inhibitors available with lower residual activity than paclobutrazol and here we have focused on two in particular. The first is the onium compound chlormequat chloride [(2-chloroethyl) trimethylammonium chloride or CCC], a GA biosynthesis inhibitor commercially used on cereals since the mid 1960s (Herbert 1982). It is readily broken down in the environment (Hampton 1988) making it a favourable choice for general application. Chlormequat chloride disrupts GA biosynthesis prior to the steps at which paclobutrazol acts, inhibiting cyclization of geranylgeranyl diphosphate to copalyl diphosphate (Davis and Curry 1991). The second GA biosynthesis inhibitor prohexadione calcium (calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate or BX-112) is a cyclohexanetrione derivative that acts much later in the GA biosynthesis pathway compared with paclobutrazol. Prohexadione binds to, and deactivates 2-oxoglutarate dependent dioxygenases (Griggs *et al.* 1991) such as 3β-hydroxylases, which convert GA_{20} to GA_1 (Nakayama *et al.* 1990). Stem and foliar applications of prohexadione can significantly reduce GA_1 levels in woody plants with no residual activity

(Nakayama *et al.* 1990, Junttila *et al.* 1991, Junttila 1993, Wang *et al.* 1995). We have undertaken an experiment to determine whether precocious flowering in *E. nitens* seedlings can be induced using the plant growth regulators (PGRs), paclobutrazol, chlormequat chloride and prohexadione and to examine if they affect growth and GA levels.

4.2 METHODS AND MATERIALS

4.2.1 Plant material

Seedlings from five seed lots (EXT 146-150) taken from three provenances: Rubicon (EXT 146); Toorongo (EXT 147, EXT 148 and EXT 150); and Northern New South Wales (EXT 149) (Eldridge *et al.* 1993) were provided by North Eucalypt Technologies, Ridgley, Tasmania. All seeds were sown in June 1995 into a mix of peat and perlite with a fine covering of vermiculite. Seedlings were pricked out three weeks later into a peat perlite mix containing 3-4 month Osmocote in individual pots (50 mm square x 120 mm deep) supported in wire frame trays, and maintained in a glasshouse until spacing was required when about 4 months old. After spacing, seedlings were hardened off in shade houses, then in outside beds. Seedlings were transferred to the Hobart campus of the University of Tasmania in April 1996 and re-potted into 55 x 50 x 175 mm PVC potting bags containing a potting mix of composted pine bark, coarse washed river sand and peat moss in a 6:4:1 ratio (v/v). The potting mix was supplemented with 1 $g.L^{-1}$ of 3-4 month Osmocote, 2 $g.L^{-1}$ 8-9 month Osmocote, 2.7 $g.L^{-1}$ dolomite lime, 0.5 $g.L^{-1}$ Micromax, 3.3 $g.L^{-1}$ iron chelate and had a pH of 6.5. The seedlings were maintained outside on the

Hobart campus of the University of Tasmania (elev. 150 m) and watered using a timed sprinkler system. An examination for flower buds and final measurements was made on the surviving seedlings at two years of age (June 1997).

4.2.2 PGR application

In mid May 1996, the 11 month-old seedlings were sprayed to run off with aqueous solutions containing either prohexadione (Kumiai Chemical Industry Co., Ltd.), chlormequat chloride (BDH Chemicals) or paclobutrazol (as "Cultar" 250 $g.L^{-1}$ paclobutrazol, ICI). There were three rates of application for each PGR: zero (control), one application of 1.705 mM a.i. (low) and two applications, one month apart, of 3.41 mM a.i. (high). All treatment solutions contained the wetting agent Tween 20 at 0.05% v/v and each PGR treatment was carried out on 51 seedlings whilst, the control group had 65 seedlings. The number of seedlings available in each seed lot was partitioned equally amongst treatments (4-16 seedlings per seed lot per treatment), with 7-20 seedlings per seed lot allocated to the control. When sprayed, the seedlings were positioned horizontally to prevent run-off into the potting medium. The seedlings were then moved to a covered shade house for 48 hours to prevent rain washing the PGR solutions off the leaves. The seedlings were then returned outside and arranged in three randomised blocks, each block comprising 17 plants of each treatment and 21-22 controls. Within treatments, seed lots were partitioned as equally as possible across replicates, and within replicates there was complete randomisation of seedlings from each seed lot and treatment. In mid-September 1996, 12 plants from the control group and from each of the high dose rate groups of the PGRs were randomly selected. The apical tissue and unexpanded leaves of these

seedlings were removed, yielding about 4 g FW from each treatment. The harvested material was immediately immersed in cold (-20°C) methanol.

4.2.3 Gibberellin analysis

The method for GA analysis was a refinement of the methods in Hasan *et al.* (1994) and Matysek (1995). Homogenised material was extracted for 23 hours at 4°C in 80% methanol, prior to filtering. Extract filtrates were supplemented with 1 ng of [17,17-$^2H_2$] GA_1 and GA_{20} (supplied by Prof. L. Mander, Research School of Chemistry, Australian National University, Canberra Australia) and 3000 dpm of [^{3}H] GA_1 (46.3 Ci/mmol) and 5600 dpm of [^{3}H] GA_{20} (30 Ci/mmol) (Amersham International, Little Chalfont, U. K.) for each g FW of plant material. The filtrate was reduced to the aqueous phase (in vacuo, <35°C) then partitioned and dried (Hasan *et al.* 1994). Dried extracts were redissolved in 10 ml of 0.4% acetic acid, filtered and washed with a further 15 ml of 0.4% acetic acid. Anion exchange was performed (Hasan *et al.* 1994) with an additional follow-up elution of the GAs with 20 ml of 0.5 M formic acid, whilst the anion exchange chromatography was carried out only once per sample.

The eluate containing the GAs was taken to dryness under vacuum and the residue redissolved in 10 ml of 60% methanol. A Waters Sep-Pak Classic C18 cartridge was preconditioned with 20ml of 60% methanol then the GA solution passed through it, followed by a rinsing with 10 ml of 60% methanol. The combined GA extracts were then reduced to dryness under vacuum.

The preparations, conditions and calculations described in Hasan *et al.* (1994) for HPLC fractionation and quantification of GA_1 and GA_{20} by GC-MS were used, with modification to only the gas chromatographic conditions. Two types of chromatographic columns were used in the analysis, an HP-1 and an HP-5 column. For the HP-1 column (0.17 μm film, 0.32 mm internal diameter x 25 m) the oven temperature was programmed for 60°C to 230°C at 30°C.min^{-1}, then to 265°C at 3°C.min^{-1}. For the HP-5 column (0.52 μm film, 0.32 mm internal diameter x 25 m) the oven temperature was programmed for 60°C to 250°C at 30°C.min^{-1}, then to 290°C at 10°C.min^{-1}.

4.2.4 Statistical analysis

The effects of replicate (1-3), seed lot (EXT 146-150), treatment (control, and high and low levels of paclobutrazol, chlormequat chloride and prohexadione) and the seed lot by treatment interaction on the increase in seedling height after treatment was examined with a restricted maximum-likelihood (REML) analysis using PROC MIXED in SAS (SAS 1992). All factors were treated as fixed effects in the model. Initial height was also included as a covariate in this model in a separate analysis to determine whether the effect of initial height on subsequent height increment affected the results. Least squares means and their standard errors were calculated for each level of the main effects and the interaction. Specific *a priori* contrasts of each PGR dose level to the control were undertaken based on the treatment main effect. Specific interactions between seed lots and each type of PGR were further explored by repeating the initial analysis with the treatment effect comprising only the control and low and high levels of each PGR.

4.3 RESULTS AND DISCUSSION

The statistical analysis showed highly significant ($p < 0.001$) effects of treatment, seed lot and their interaction on height increment. However, the effect of the seed lot by treatment interaction was small compared to the main effects and due mainly to complex differential growth responses of the seed lots to chlormequat chloride ($p < 0.001$). In contrast, the seed lot by treatment interaction for paclobutrazol and prohexadione was small ($p < 0.05$) and the direction of the growth response was consistent across seed lots. The effect of initial height on height increment was significant ($p < 0.001$), but did not change the overall result of the analysis.

Figure 4.1 shows clearly the relative effectiveness of the different PGRs in reducing growth and GA_1 and GA_{20} levels. Paclobutrazol was found to be the most effective PGR at reducing the levels of both GAs tested and the growth rate, being the only PGR to reduce growth significantly at both levels used. The growth retarding effects of chlormequat chloride are know to be less effective than paclobutrazol (Davis and Andersen 1989), and the results here were consistent with that (Figure 4.1). However, it should be noted that the response of the *E. nitens* seed lots to the chlormequat chloride was complex and difficult to interpret, with some seed lots responding positively to the low dose but negatively to the high dose. In seedlings of *Salix pentandra*, prohexadione had similar activity to chlormequat chloride in reducing growth (Junttila *et al.* 1991). However, prohexadione had no significant effect on growth at the levels used and was less effective that chlormequat chloride here. Because of its low residual activity, regular application of prohexadione may be required to be efficacious (Junttila *et al.* 1991, Rademacher *et al.* 1992, Junttila 1993).

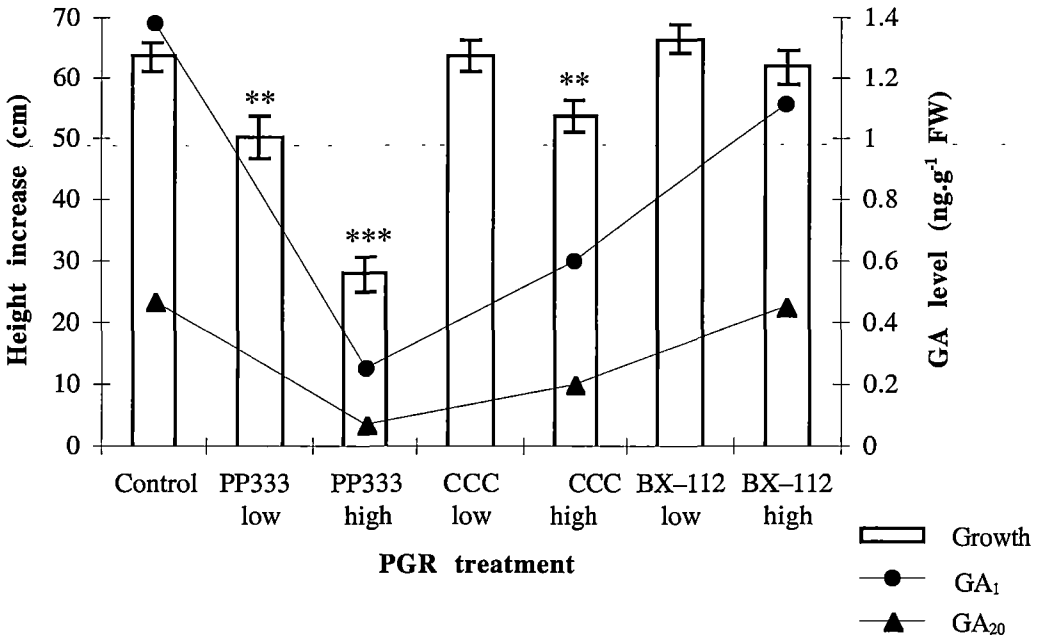

Figure 4.1 The means (±se) of the height increase of the seedlings in all treatments and the GA_1 and GA_{20} levels for the control seedlings and seedlings treated with the high dose of PGRs. Asterisks denote level the of significant difference of the mean heights for the treatments to the control mean height based on specific contrasts in the mixed model analysis. Key: ** = p <0.01, *** = p <0.001.

In the apical tissue of the seedlings, the degree to which GA_1 and GA_{20} levels were reduced by paclobutrazol was similar, around 83%, and this was also the case for chlormequat chloride where they were both reduced by 57% (Figure 4.1). However, prohexadione differentially affected levels of GA_1 compared to GA_{20} consistent with (Nakayama *et al.* 1990). Prohexadione reduced GA_1 levels by 20%, whilst GA_{20} levels were reduced by only 4% (Figure 4.1). It could be expected that inhibition of GA_1 biosynthesis may lead to GA_{20} levels increasing rather than a decrease as found. The excess GA_{20} may have been converted to GA_{29}, as increased levels of GA_{29} have been found in wheat, barley and oilseed rape treated with the prohexadione analogue LAB 236 735 (Rademacher *et al.* 1992). The early 13-hydroxylation pathway has been suggested

as the dominant GA biosynthetic pathway in *E. nitens* (Hasan *et al.* 1994), and the apparent action of prohexadione in this experiment supports this claim. The GA_1 levels in the controls were twice that found previously in similar tissues (Hasan *et al.* 1994) and may be the result of seasonal fluctuations as found in *E. globulus* (Matysek 1995).

Flower buds were found on only four seedlings, three from seed lot EXT 149 treated with the low rate of paclobutrazol and one from seed lot EXT 148 treated with the high rate of chlormequat chloride. All other seedlings remained vegetative throughout the experiment. The low occurrence of the buds does not allow for analysis to determine if this was a consequence of the treatments or naturally occurring precocious flowering.

Although the paclobutrazol treatment applied to the *E. nitens* seedlings, and the conditions under which they were kept, were similar to those reported to induce precocious flowering in *E. globulus* by (Hasan and Reid 1995), none developed flower buds. This re-enforces the suggestion that there is a large inter-specific difference in the ability to induce precocious flowering (Griffin *et al.* 1993). However, the reproductive response of mature *E. nitens* to paclobutrazol was found to be similar to that of *E. globulus* (Griffin *et al.* 1993). Flower buds have been induced by paclobutrazol in the season following treatment of 6 month old grafts of mature *E. nitens* scions on seedling rootstock (Moncur and Hasan 1994). This suggests that the barrier to precocious flowering in seedlings of *E. nitens* resides in the aerial part of the plant (i.e. juvenile leaves, stems and/or meristems). Moncur and Hasan (1994) produced a relationship between flower bud production and apical GA_1 levels in mature *E. nitens*. The GA_1 levels found in the chlormequat chloride and paclobutrazol treatment, were sufficiently low to produce flower buds based on their findings. Applying higher dose rates of the PGRs, particularly paclobutrazol would therefore not be expected to increase the likelihood of inducing precocious flowering but

may result in detrimental stunting of growth or phytotoxicity (Hamid and Williams 1997). An obligate requirement for an extensive cold period (analogous to vernalisation) after paclobutrazol treatment to produce flower buds in *E. nitens* grafts has been identified (Moncur and Hasan 1994). Here, the seedlings were exposed to a full winter, sufficient for nearby mature *E. nitens* trees to produce flower buds. The stimuli applied to the seedlings could then be considered to be sufficient to promote flower bud production. It therefore appears that the apical meristem is not receiving the stimulus or is unable to respond to it at such an early age.

Although paclobutrazol is far more effective in reducing GA levels in *E. nitens* seedlings than either chlormequat chloride or prohexadione, the application of paclobutrazol was not sufficient to induce precocious flowering as occurs in *E. globulus* (Hasan and Reid 1995). This implies that an extra level of reproductive inhibition may be operating within the juvenile phase of this species and a more complex set of conditions must be satisfied before reproductive development is initiated.

Chapter 5

The effect of fertiliser and paclobutrazol on flowering precocity and abundance

5.1 INTRODUCTION

In adult *Eucalyptus nitens* trees, application of paclobutrazol by soil drenching of the root zone vastly increases the abundance and reliability of the flower bud crop and subsequent seed harvest with no significant degradation in seed quality detected to date (Griffin *et al.* 1993). However, at higher application rates, paclobutrazol results in reduced leaf size (Hetherington and Jones 1990) and number (Moncur and Hasan 1994). The resulting net reduction in photosynthetic capacity coupled with the high demand for assimilates by the large bud crop, tends to reduce seeds per capsule and the ability to maintain a high rate of flower bud initiation in subsequent seasons (Griffin *et al.* 1993). Additional cultural treatments may ameliorate these side effects to some extent and increase seed production (Bonnet-Masimbert and Webber 1995).

In *E. globulus,* paclobutrazol was effective in promoting precocious flowering, reducing the generation interval by 50% (Hasan and Reid 1995). These flower buds developed whilst the plants were vegetatively juvenile (most *Eucalyptus* species are heteroblastic) which indicated sexual maturity is de-coupled from vegetative maturity (Hasan and Reid 1995). However only a small proportion of *E. nitens* seedlings have been induced to produce buds by paclobutrazol (Moncur 1998). Use of alternate growth regulators (CCC and prohexadione) in *E. nitens* did not produce a significant flowering response (Chapter

4). The evidence suggests further manipulation of chemical and/or environmental conditions is necessary in any future attempts at inducing substantial levels of precocious flowering in *E. nitens*.

Precocious flowering in plants has been achieved by a number of non-hormonal cultural treatments including girdling, root pruning, extended photoperiod, water stress and fertilisation (Chalupka and Cecich 1997). However, it is still not clear what mechanism(s) mediates this response (Bonnet-Masimbert and Webber 1995). One well supported theory proposes that plants must reach a minimum size before reproductive growth can be initiated, an evolutionary development to improve the chances of successful early establishment (Bernier *et al.* 1981). Treatments to accelerate early growth (extended photoperiod and fertilisation) would reduce the time taken for plants to reach this minimum size and consequently first flowering (Hackett 1985). Cameron and Kube (1983) found that application of a nitrogen and phosphorus containing fertiliser increased the number of *E. regnans* trees flowering in the first season. The increases in flower production was attributed to tree size, the fertiliser treatment had promoted rapid growth and as a consequence decreased the mean time to first flowering.

Fertiliser application is also beneficial to flowering abundance in mature trees with nitrogen considered to be most efficacious (Sedgley and Griffin 1989). In mature radiata pine, application of nitrogen fertiliser improved flowering and seed yields substantially (Griffin *et al.* 1984). However, Griffin *et al.* (1984) could not determine if this affect was due to a florigenic effect of nitrogen or simply related to canopy growth. There is a considerable draw-back to the use of fertilisers in seed orchards in terms of crown management. Rapid and extensive canopy growth impedes access to flowers and capsules requiring more intensive use of limb bracing, pollarding and pruning in association with personnel elevating equipment (Eldridge *et al.* 1993).

The application of nitrogen fertiliser with paclobutrazol has shown to benefit flowering whilst maintaining desirable canopy form compared to applying them singularly in peach (George and Nissen 1992) and pear (Raese and Burts 1983). Similarly, in Douglas-fir where $GA_{4/7}$ is applied to induce flowering, co-application with nitrogen fertiliser yielded more flower buds than when either was applied alone (Daoudi *et al.* 1994). Potentially, a synergistic response may be produced by combinations of hormonal and cultural treatments exerting influence at different points in the flowering mechanism (Bonnet-Masimbert and Webber 1995). Economically, the application of fertiliser, which is relatively cheap, may enhance the effectiveness of paclobutrazol which is relatively expensive, reducing seed production costs.

This chapter examines the effects on *E. nitens* trees of (1) fertilisation with nitrogen and phosphorus on (a) the first two flowering seasons (reproductive maturity) and (b) vegetative phase change (vegetative maturity) and (2) combining nitrogen fertilisation with the application of paclobutrazol, on the initiation and abundance of flower buds on (a) juvenile (< 4 years old) and (b) mature (> 4 years old) trees. Throughout this examination of foliage and reproductive traits, their relationships to tree growth are also explored.

5.2 METHODS AND MATERIALS

5.2.1 Fertilisation with nitrogen and phosphorus

5.2.1.1 Site description

Two *E. nitens* fertiliser trials which had been established by the CRC-THF were examined for the effects of nitrogen and phosphorus fertilisation on flower bud production. These trials were located at Tim Shea in southern-central Tasmania and at Nunamara in the north-east of Tasmania and are described in detail in Wang *et al.* (1998). After clearing and windrowing, the sites were ripped to a depth of *ca.* 70 cm and mounded at 4 m intervals. The *E. nitens* seedlings for both sites were raised from open pollinated seed taken from Boral Tasmania, Camden seed orchard in north-east Tasmania. The Tim Shea site was planted in September 1993 whilst the Nunamara site was planted in October 1993. Both sites were planted with paper tube stock at a within row spacing of 2 m and an inter-row spacing of 4 m. Two months after planting, all seedlings were given an establishment lump spot dose of 10:17:8 N:P:K at a rate of 200 g per plant at Nunamara and 100 g per plant at Tim Shea. Experimental blocks were established as 5 by 5 trees with two tree buffers separating each block. Intensive weed control was employed through the use of chemical control in the first 18 months and mechanical slashing from then on prior to fertiliser applications. The fertiliser treatments were applied factorially at 4 levels of nitrogen (N1-N4) as urea (46% N) and 3 levels of phosphorus (P1-P3) as triple superphosphate (20% P). The fertiliser was applied over three consecutive years and treatments are summarised in Table 5.1. A separate timing of treatment regime was established where a single dose rate of nitrogen (200 $kg.ha^{-1}$) as urea was applied at either one (N5), two (N6) or three (N7) years after planting. All treatment combinations (both

factorial and timing) were randomised and replicated three times at each site. All fertilisers were applied by hand broadcast.

Table 5.1 Dose rates and the timing of application after planting of nitrogen (applied as urea) and phosphorus (applied as triple super phosphate) fertiliser applied to *E. nitens* trees growing at Tim Shea and Nunamara.

		Year and rate ($kg.ha^{-1}$) of fertiliser application			
Fertiliser	Dose code	Year 1	Year 2	Year 3	Total
Nitrogen	N1	0	0	0	0
	N2	25	50	125	200
	N3	50	100	150	300
	N4	100	200	200	500
Phosphorus	P1	0	0	0	0
	P2	75	0	0	75
	P3	150	0	0	150

5.2.1.2 Trial assessment

All growth (height and diameter) data for the Tim Shea and Nunamara trials were collected and kindly made available by staff of the Sustainable Management Program of the CRC-SPF (formally the CRC-THF). In July 1995 (*ca.* 2.5 years old) and in May 1996 (*ca.* 3.5 years old), all experimental trees were directly measured for diameter over bark at breast height and height. In June 1997 (*ca.* 4.5 years old) all experimental trees at both sites were measured only for diameter over bark at breast height, the individual heights were calculated based on regressions derived from the heights and diameters of 5 tree diameter classes per treatment plot for all treatments at each site, each site with its own regression (R. Cromer pers. comm.).

Eucalyptus nitens produces umbels of seven flower buds in the axials of new growth in the spring, following initiation which is stimulated by a cold winter (Moncur and Hasan 1994) and are not visible to the naked eye on close inspection until two to three months following initiation (Moncur *et al.* 1994b). In a plantation, the umbels are at their largest and most conspicuous approximately 12 months after initiation, prior to both the spring flush of growth which can obscure them and flowering where whole umbels can be lost if all their flowers are not pollinated within a few weeks. Consequently, the assessment of umbels was made in August 1996 (*ca.* 3.5 years old) and September 1997 (*ca.* 4.5 years old). The number of umbels per tree was based on a visual estimate of the actual number of umbels. To examine if the timing of phase change (vegetative maturity) was affected by fertiliser treatment, the amount of adult foliage on each tree was determined during the 1996 umbel assessment by estimating the percentage of tree height of which the canopy bore fully adult foliage (petiolate leaves) (Jordan *et al.* 1999).

5.2.1.3 Statistical analysis

Statistical models were fitted to the data for the factorial and timing application experiments using block means for the traits of height, diameter, proportion of trees with umbels, proportion of trees with adult foliage and proportion of tree height which bore adult foliage. Individual tree data were used for the trait of number of umbels per tree and analysis was restricted to only those trees with umbels. Analyses were done on the combined data for both sites and then on the data from Tim Shea and Nunamara separately if necessary.

For the data from the factorial experiment across sites, the model fitted was:

trait = mean + site + rep(site) + N + P + N*P + N*site + P*site + N*P*site + error.....(Eqn. 5.1),

where site is the fixed location (Tim Shea or Nunamara) effect, rep(site) is the random effect of replicate (1-3) within site, N is the fixed effect of nitrogen (N1 - N4), P is the fixed effect of phosphorus (P1 - P3) and the remaining terms are the fixed two and three way interactions. The rep(site) effect was used to obtain an approximate test of the significance of the site effect, whereas the significance of all other terms was tested against the error. Analyses were also undertaken to examine and remove the effect of tree size in the specific trait by including either height or diameter as a covariate and a covariate by site interaction in Eqn. 5.1.

In the covariate analysis of proportion of trees with umbels, the growth data from the year previous to when the umbel data was collected was used as these measurements were taken close to the time of initiation. However, in the covariate analysis for the number of umbels per tree, growth measurements from the same year as the umbel data were used as the covariate as this better represents the number of available axials from which umbels would potentially develop. In the factorial experiment, only the Tim Shea site had sufficient trees with umbels in 1996 to permit reasonably balanced analysis of the number of umbels per tree.

Individual site analyses of the factorial experiment were undertaken using the statistical model:

trait = mean + rep + P + N + N*P + error.....(Eqn. 5.2),

where rep is the random effect of replicate (1-3), N is the fixed effect of nitrogen (N1 - N4), P is the fixed effect of phosphorus (P1 - P3) and N*P is the fixed two way interaction of nitrogen and phosphorus. As in the combined site analysis, tree size effects for the appropriate year were accounted for by the addition of a covariate term to Eqn. 5.2.

For the timing experiment the model fitted to the combined site data was:

trait = mean + site + rep(site) + N + N*site + error.....(Eqn. 5.3),

where site is the fixed site (Tim Shea or Nunamara) effect, rep(site) is the random effect of replicate (1-3) within site, N is the fixed effect of the time of nitrogen application (N1, N5 - N7) and N*site is the fixed two way interaction of nitrogen with site. The rep(site) effect was used to obtain an approximate test of the significance of the site effect, whereas the significance of all other terms was tested against the error. Again, tree size and the interaction of tree size with location effects for the appropriate year were accounted for by the addition of a covariate and covariate by site term to Eqn. 5.3.

Individual site analyses of the timing experiment were undertaken using the statistical model:

trait = mean + rep + N + error.....(Eqn. 5.4),

where rep is the random effect of replicate (1-3), N is the fixed effect of nitrogen (N1, N5 - N7). Size effects were accounted for by the addition of a covariate term to Eqn. 5.4 as necessary.

Specific contrasts between the treatment levels of each main effect (N and P) were undertaken and least square means of the main effects and their combinations calculated for each trait. Where necessary, transformations of the raw data were undertaken to optimise the normality of the residuals and homogeneity of the variances. For presentation of the data for transformed traits, the least squares means and their standard error limits were back transformed for plotting, resulting in asymmetric standard errors. The statistical models were initially fitted with the PROC GLM procedure in SAS (SAS 1992) to examine the need to transform the raw data. The models were then fitted again to the data (which was transformed as necessary) using the PROC MIXED procedure in SAS (SAS 1992) to generate the specific contrasts, least squares means and standard errors.

5.2.2 Fertilisation with nitrogen and co-application of paclobutrazol

5.2.2.1 The effect on flower initiation and abundance in juvenile (< 4 years old) trees

Site description and treatment

The trial site was located near Hampshire in north-west Tasmania and is 4 km west of the Basils-2 site described in Wang *et al.* (1998) and previously planted with *Pinus radiata.* After clearing and windrowing, the site was ripped and mounded at 3.5 m intervals. The site was planted in October to November 1994 at a within row spacing of 2.6 m and inter-row spacing of 3.5 m. The genetic material consisted of genets of two clones "clone 1" and "clone 2", propagated as cuttings six to eight months earlier by North Eucalypt Technologies, Ridgley and planted in adjacent clonal blocks. Within two months of planting, all genets were given 100 g of 18:20:0 DAP fertiliser by a spade slit. Treatment

blocks were established in November 1996 (*ca.* 2.5 years old) as 2 by 2 trees with a minimum of 2 tree buffers separating blocks. Trees were measured for height and stem diameter 15 cm from ground level. Most trees were between 1 and 2 m in height and all were vegetatively juvenile and on close inspection, no umbels were evident. Treatments followed a factorial combination of three levels of nitrogen; 0 (control), 50 and 150 $kg.ha^{-1}$ by three levels of paclobutrazol; 0 (control), 0.3 and 1.0 g per cm stem circumference ($g.cm.circ^{-1}$) at 15 cm height (Griffin *et al.* 1993) for a total of nine different treatments. The nitrogen was applied as high biuret urea (46% N) via a spade slit *ca.* 20 cm from the base of the tree in November 1996. The paclobutrazol as Cultar (250 $g.L^{-1}$ paclobutrazol, ICI) was dispersed in 1 litre of water and applied as a collar drench in February 1997. A complete randomised block design was used with seven replicates across clone 1 and eight replicates across the adjacent clone 2. The trial was measured for height and stem diameter 15 cm above the ground and the number of umbels per tree counted in May 1998 (*ca.* 4 years old).

Statistical analysis

A statistical model was fitted to the data using block means for the traits of height, diameter and proportion of trees with flower buds whilst individual tree data was used for the trait of number of umbels per tree which was restricted to only those trees with umbels and treated with both nitrogen and paclobutrazol. This was done on the combined data for both clones and then on the data from each clone separately. For the combined clone data, the model fitted was:

trait = mean + cl + rep(cl) + N + pac + N*pac + N*cl + pac*cl + N*pac*cl + error...(Eqn. 5.5),

where cl is the fixed clone (clone 1 or clone 2) effect, rep(cl) is the random effect of replicate (1-7/8) within clone, N is the fixed effect of nitrogen (0, 50, 150), pac is the fixed effect of paclobutrazol (0, 0.3, 1.0) and the remaining terms are the fixed two and three way interactions. The rep(cl) effect was used to obtain an approximate test of the significance of the clone effect, whereas the significance of all other terms was tested against the error. Analyses were also undertaken to examine and remove the effect of tree size on the specific trait by including either height or diameter as a covariate in Eqn. 5.5. In this case, either initial height and diameter was used as a covariate for the analysis of the proportion of trees with umbels, and final height and diameter as covariates when analysing for the number of umbels per tree.

Analyses of each clone were undertaken using the statistical model:

trait = mean + rep + N + pac + N*pac + error.....(Eqn. 5.6),

where rep is the random effect of replicate (1-7/8), N is the fixed effect of nitrogen (0, 50, 150), pac is the fixed effect of paclobutrazol (0, 0.3, 1.0) and N*pac is the fixed two way interaction. Similarly, tree size effects were accounted for by the addition of a covariate term to Eqn. 5.6.

Models were fitted to the data to produce specific contrasts, least squares means and standard errors for the main effects of nitrogen and paclobutrazol in SAS (SAS 1992) as described for section *5.2.1.3*. Pearsons correlations between growth and reproductive traits were carried out using the PROC CORR procedure in SAS (SAS 1992).

5.2.2.2 The effect on flower initiation and abundance in adult (> 4 years old) trees.

Site description and treatment

The trial site was located adjacent to the Nunamara site described in section *5.2.1.1* and was part of the initial establishment but not utilised further. There was a distinct change in soil colour across this site. In the northern half, the soil was grey in colour whilst in the southern half the soil was a red-brown colour. Experimental blocks were established in February 1997 (*ca.* 4 years old) as 2 by 2 trees with a minimum of 2 tree buffers separating blocks. For each tree, an initial measurement of height and diameter over bark at breast height was made prior to treatment. Treatments were applied as factorial combinations of two levels of nitrogen; 0 and 300 $kg.ha^{-1}$ by two levels of paclobutrazol; 0 and 1.0 $g.cm.circ^{-1}$ at breast height (Griffin *et al.* 1993). The nitrogen was applied as high biuret urea (46% N) broadcast spread by hand whilst the paclobutrazol as Cultar (250 $g.L^{-1}$ paclobutrazol, ICI) was dispersed in 1 litre of water and applied as a collar drench around each tree. Treatments were randomised in complete blocks and replicated 7 times on each soil type. The experimental trees were measured for height, diameter at breast height and the actual number of umbels per tree visually estimated in October 1998 (*ca.* 5.5 years old).

Statistical analysis

The data for the effect of nitrogen and paclobutrazol on block means for the traits of height, diameter, presence and the number of flower buds for combined soil types were analysed as in section *5.2.2.1* using Eqn. 5.5, where clone was replaced with soil type in

the model. As in section *5.2.2.1*, the main effects on each trait on each soil type were found using Eqn. 5.6. The raw data was applied to the models as described in section *5.2.1.3* using PROC MIXED in SAS (SAS 1992) to derive specific contrasts, least squares means and standard errors for each trait.

5.3 RESULTS

5.3.1 Fertilisation with nitrogen and phosphorus

5.3.1.1 The effect on the first two seasons of flower bud production (reproductive maturity)

Factorial experiment

Neither nitrogen or phosphorus treatments had significant main effects on the percentage of trees with umbels in either 1996 or 1997 (Table 5.2). However, nitrogen significantly increased the number of umbels per reproductive tree in 1997 (Table 5.2). Nitrogen also significantly increased growth rate in 1997 (Table 5.2) and, when either height or DBH was included as a covariate, the overall effect of nitrogen was no longer significant on the number of umbels per reproductive tree, but a significant nitrogen by site interaction was revealed (Table 5.3). This interaction was due to nitrogen significantly increasing the number of umbels per reproductive tree at Tim Shea but not at Nunamara independently of growth (Table 5.4, Figure 5.1). These effects were not expressed in 1996 and the impact of nitrogen fertilisation on growth increased with age (Table 5.2).

In 1997, Nunamara had a significantly greater proportion of trees with umbels (39.2%) compared to Tim Shea (25.5%) (Table 5.2). In contrast, mean umbel abundance per reproductive tree in 1997 at Tim Shea was 65.3 which was significantly higher that at Nunamara which had 39.0 umbels per reproductive tree (Table 5.2). However, the differences between the sites for both the percentage of trees with umbels and number of umbels per reproductive tree were solely due to growth rate. Tree diameter was significantly affected by site in 1997 (Table 5.2), with trees at Tim Shea of greater diameter than those at Nunamara. When the respective height or diameter measurements (i.e. from 1996 for the percentage of trees with umbels and from 1997 for the number of umbels per reproductive tree) were included as a covariate, both were significant for each trait whilst the effect of site was no longer significant (Table 5.3).

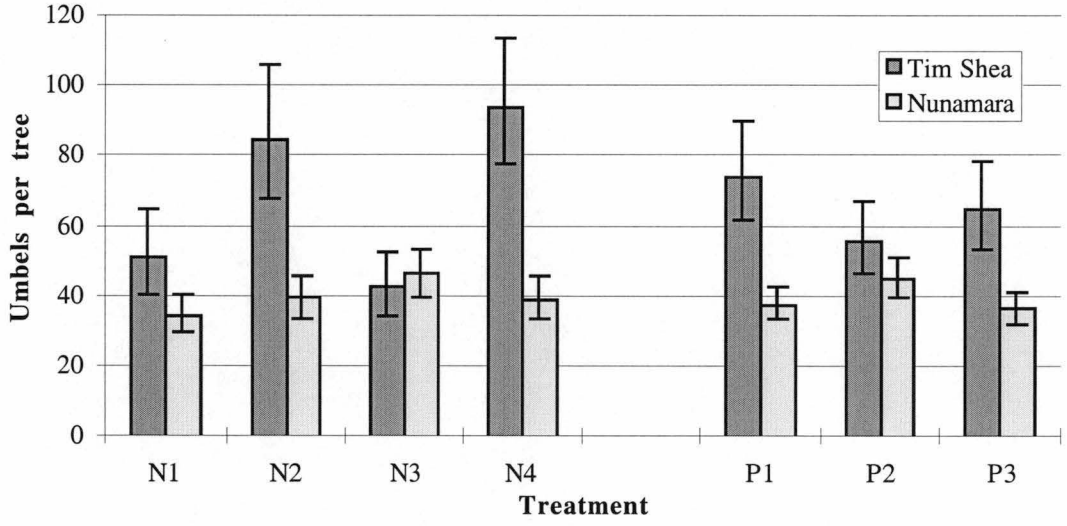

Figure 5.1 Least squares means (±se) of the number of umbels per reproductive tree (with tree diameter fitted as a covariate) at Tim Shea and Nunamara in 1997 (4.5 years old) in response to fertilisation with nitrogen (N1 - N4) and phosphorus (P1 - P3). For treatment dose rates and the timing of their application refer to Table 5.1.

Table 5.2 ANOVA table for the effect of trial site, nitrogen (N) and phosphorus (P) fertiliser on reproductive, foliage and growth traits in trials at Tim Shea and Nunamara in 1996 (3.5 years old) and 1997 (4.5 years old). Refer to Table 5.1 for fertiliser dose rates and time of application. F values and levels of significance are shown. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001. [a]The error degrees of freedom given are only for analyses based on plot means. [b]Analysis of the number of umbels per tree was based on individual tree data for only those trees with umbels, error degrees of freedom for all fertiliser effects = 47.

Effect	*Site*	*N*	*P*	*N*P*	*N*Site*	*P*Site*	*N*P*Site*
DF	1	3	2	6	3	2	6
Error DF[a]	4	44	44	44	44	44	44
% of trees with umbels in 1996	2.26 n.s.	1.21 n.s.	2.03 n.s.	0.87 n.s.	0.44 n.s.	1.64 n.s.	1.16 n.s.
% of trees with umbels in 1997	11.16 *	2.73 n.s.	0.58 n.s.	0.28 n.s.	0.12 n.s.	0.43 n.s.	0.37 n.s.
Number of umbels per tree in 1997[b]	14.58 ***	3.38 *	0.51 n.s.	0.40 n.s.	2.74 n.s.	1.32 n.s.	0.95 n.s.
% of trees with adult foliage in 1996	0.13 n.s.	2.94 *	3.22 *	0.65 n.s.	1.75 n.s.	0.65 n.s.	1.19 n.s.
% of canopy with adult foliage in 1996	1.10 n.s.	2.70 n.s.	4.16 *	0.26 n.s.	0.83 n.s.	0.57 n.s.	1.39 n.s.
Height in 1995	1.64 n.s.	1.62 n.s.	0.09 n.s.	0.23 n.s.	0.19 n.s.	0.64 n.s.	0.91 n.s.
Height in 1996	1.68 n.s.	2.70 n.s.	0.02 n.s.	0.45 n.s.	0.27 n.s.	0.44 n.s.	0.77 n.s.
Height in 1997	0 n.s.	12.44 ***	0.56 n.s.	1.17 n.s.	0.24 n.s.	0.65 n.s.	1.54 n.s.
DBH in 1995	0.21 n.s.	2.36 n.s.	0.27 n.s.	0.32 n.s.	0.29 n.s.	0.30 n.s.	1.03 n.s.
DBH in 1996	0.09 n.s.	6.37 **	0.01 n.s.	0.83 n.s.	0.22 n.s.	0.66 n.s.	1.43 n.s.
DBH in 1997	13.00 *	12.56 ***	0.59 n.s.	1.25 n.s.	0.03 n.s.	0.71 n.s.	1.57 n.s.

Table 5.3 ANOVA table for the effects of trial site, nitrogen (N) and phosphorus (P) fertiliser on reproductive and growth traits in trials at Tim Shea and Nunamara in 1996 (3.5 years old) and 1997 (4.5 years old) with growth traits (height or DBH) measured in year shown included as a covariate. Refer to Table 5.1 for fertiliser dose rates and time of application. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. [a]The error degrees of freedom given are only for analyses based on plot means. [b]Analysis of the number of umbels per tree was based on individual tree data for only those trees with umbels, error degrees of freedom for main effects = 47 whilst covariate effects = 449.

	Effect	*Covariate*	*Covariate* Site*	*Site*	*N*	*P*	*N*P*	*N*Site*	*P*Site*	*N*P*Site*
	DF	1	1	1	3	2	6	3	2	6
	Error DF[a]	42	42	4	42	42	42	42	42	42
	Covariate									
% of trees with umbels in 1996	Height in 1995	13.64 ***	0.03 n.s.	0.14 n.s.	0.40 n.s.	2.14 n.s.	0.87 n.s.	0.61 n.s.	1.02 n.s.	0.81 n.s.
% of trees with umbels in 1996	DBH in 1995	18.45 ***	0.22 n.s.	0.03 n.s.	0.33 n.s.	2.19 n.s.	0.95 n.s.	0.62 n.s.	1.43 n.s.	1.19 n.s.
% of trees with umbels in 1997	Height in 1996	11.39 **	0.18 n.s.	0.01 n.s.	1.78 n.s.	0.67 n.s.	0.23 n.s.	0.13 n.s.	0.78 n.s.	0.59 n.s.
% of trees with umbels in 1997	DBH in 1996	11.27 **	0.10 n.s.	0.03 n.s.	0.78 n.s.	0.63 n.s.	0.28 n.s.	0.09 n.s.	0.98 n.s.	0.55 n.s.
Number of umbels per tree in 1997[b]	Height in 1997	39.27 ***	0.25 n.s.	0.00 n.s.	1.85 n.s.	0.20 n.s.	0.61 n.s.	3.26 *	1.15 n.s.	1.20 n.s.
Number of umbels per tree in 1997[b]	DBH in 1997	40.58 ***	0.07 n.s.	0.35 n.s.	1.85 n.s.	0.20 n.s.	0.61 n.s.	3.22 *	1.12 n.s.	1.18 n.s.

Table 5.4 ANOVA table for individual trial site analyses based on single tree data of the effects of nitrogen (N) and phosphorus (P) fertiliser on the number of umbels per reproductive tree in 1997 (4.5 years old) with either tree height or DBH in that year included as a covariate. Refer to Table 5.1 for fertiliser dose rates and time of application. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001.

	Effect	*Covariate*	*N*	*P*	*N*P*
	DF	1	3	2	6
Tim Shea	**Error DF**	178	23	23	23
	Covariate				
Number of umbels per tree in 1997	Height in 1997	10.41 **	3.41 *	0.61 n.s.	0.75 n.s.
Number of umbels per tree in 1997	DBH in 1997	10.67 **	3.39 *	0.60 n.s.	0.74 n.s.

	Effect	*Covariate*	*N*	*P*	*N*P*
	DF	1	3	2	6
Nunamara	**Error DF**	271	24	24	24
	Covariate				
Number of umbels per tree in 1997	Height in 1997	47.48 ***	0.62 n.s.	0.80 n.s.	0.85 n.s.
Number of umbels per tree in 1997	DBH in 1997	45.04 ***	0.62 n.s.	0.77 n.s.	0.85 n.s.
		271	24	24	24

Timing experiment

The timing of nitrogen application and its interaction with site, with and without growth traits as covariates, had no significant effect (p >0.05) on the number of trees with umbels in 1996 or 1997, the number of umbels per reproductive tree in 1997 or, the height and diameter of trees in 1995, 1996 and 1997. There were too few reproductive trees in 1996

to perform a balanced analysis on umbel abundance per tree. Height and diameter, both in 1995 and 1996 significantly affected the proportion of trees with umbels in 1996 and 1997 respectively ($p < 0.05$ for both covariates in each year). Similarly, height and diameter of trees in 1997 significantly affected the number of umbels per tree in 1997 ($p < 0.001$ for both covariates). This again shows a positive relationship between growth and reproductive development.

5.3.1.2 Vegetative phase change (vegetative maturity)

Factorial experiment

In 1996, both nitrogen and phosphorus significantly affected the number of trees with adult foliage whilst only phosphorus significantly affected the percentage of tree canopy which had adult foliage (Table 5.2). Growth rate itself had a significant effect on the vegetative phase change (Table 5.5) with faster growing trees making the transition to adult foliage earlier. When either tree height or diameter in 1996 was included as a covariate in the analysis for the percent of trees with adult foliage, the effect of nitrogen was no longer significant whilst the effects of both covariates (positive) and phosphorus were highly significant (Table 5.5). Overall, 25% more trees treated with the high dose of phosphorus developed adult foliage compared to controls (Figure 5.2). When either tree height or diameter in 1996 was included as a covariate in the analysis for the percentage of tree canopy which had adult foliage, both covariates and phosphorus were highly significant (Table 5.5). The proportion of tree height which was adult foliage increased from 4.2% in the controls to 7.7% in the high dose rate of phosphorus (Figure 5.3).

Table 5.5 ANOVA table for the effects of trial site, nitrogen (N) and phosphorus (P) fertiliser on the proportion of trees with adult foliage and the proportion of tree height with adult foliage that was adult in trials at Tim Shea and Nunamara in 1996 (3.5 years old) with either height or DBH measured in that year included as a covariate. Refer to Table 5.1 for fertiliser dose rates and time of application. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001.

	Effect	*Covariate*	*Covariate *Site*	*Site*	*N*	*P*	*N*P*	*N*Site*	*P*Site*	*N*P*Site*
	DF	1	1	1	3	2	6	3	2	6
	Error DF	42	42	4	42	42	42	42	42	42
	Covariate									
% of trees with adult foliage in 1996	Height in 1996	49.77 ***	3.50 n.s.	1.90 n.s.	0.89 n.s.	8.64 ***	0.65 n.s.	2.51 n.s.	1.43 n.s.	2.20 n.s.
% of trees with adult foliage in 1996	DBH in 1996	36.77 ***	2.97 n.s.	2.20 n.s.	0.19 n.s.	7.06 **	0.65 n.s.	2.94 *	0.56 n.s.	1.53 n.s.
% of canopy with adult foliage in 1996	Height in 1996	61.63 ***	3.72 n.s.	1.49 n.s.	0.65 n.s.	12.42 ***	0.50 n.s.	1.10 n.s.	1.43 n.s.	3.29 **
% of canopy with adult foliage in 1996	DBH in 1996	49.96 ***	3.97 n.s.	2.36 n.s.	0.40 n.s.	10.47 ***	0.85 n.s.	1.55 n.s.	0.53 n.s.	2.38 *

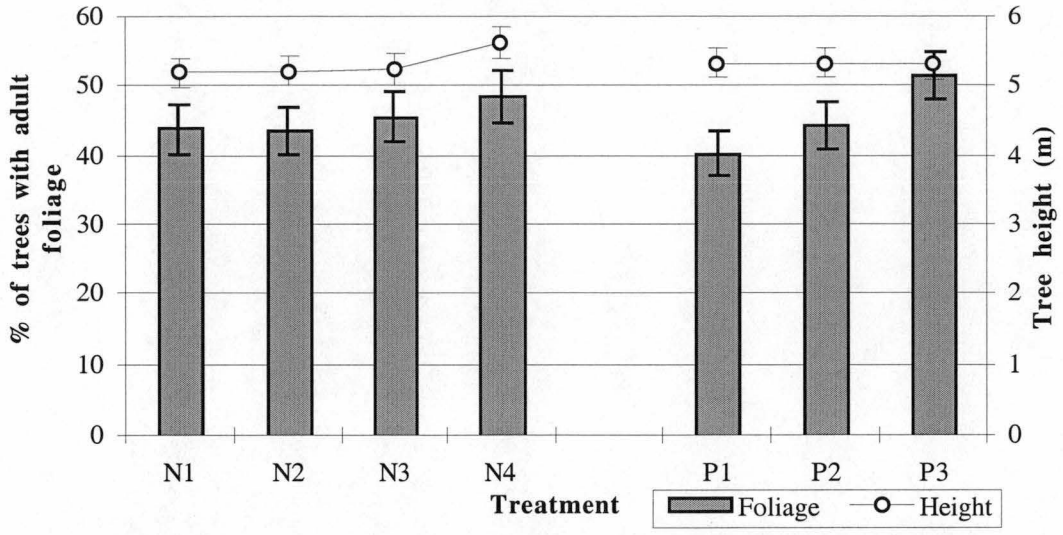

Figure 5.2 Least squares means (±se) of the proportion of trees with adult foliage (with tree height fitted as a covariate) and tree height at both Tim Shea and Nunamara in 1996 (3.5 years old) in response to fertilisation with nitrogen (N1 - N4) and phosphorus (P1 - P3, refer to Table 5.1 for application rates). Tree height data supplied by staff of the Sustainable Management Program of the CRC-SPF.

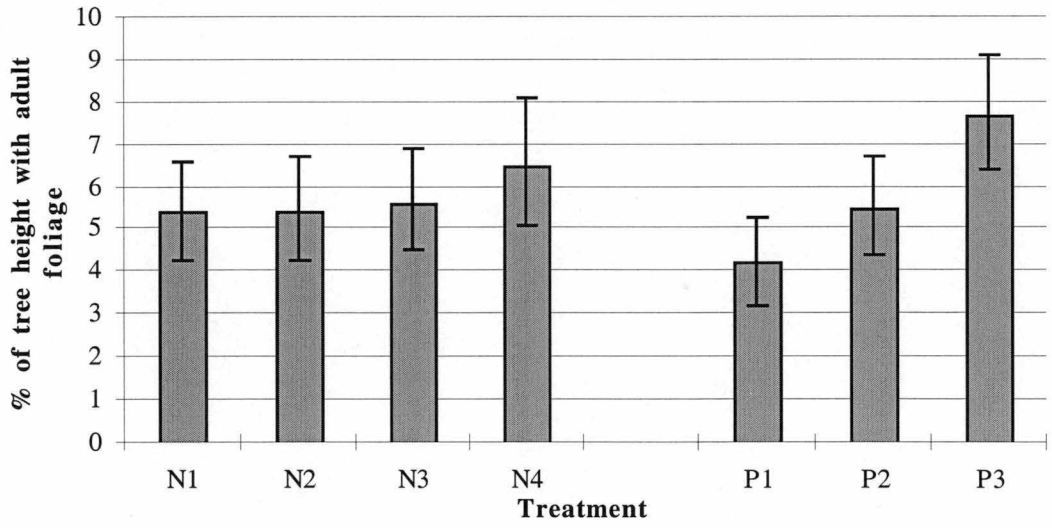

Figure 5.3 Least squares means (±se) for the amount of adult foliage on trees (with tree height fitted as a covariate) at both Tim Shea and Nunamara in 1996 (3.5 years old) in response to fertilisation with nitrogen (N1 - N4) and phosphorus (P1 - P3, refer to Table 5.1 for application rates). The amount of adult foliage is expressed as a percentage of tree height from phase change to the top of the tree.

There were slightly significant interactions between the fertiliser treatments and site in both the number of trees with adult foliage and proportion of tree canopy which was adult when DBH was used as a covariate (Table 5.5). When broken down into individual sites, the magnitude of the effects were different but the overall trends were the same at both sites (Table 5.6). When tree height was used as a covariate in the analysis of the proportion of tree canopy which was adult at each site a slightly significant interaction ($p < 0.05$) between nitrogen and phosphorus was found only at Nunamara (Table 5.6). However, this effect was not significant when tree diameter was used as the covariate (Table 5.6).

Table 5.6 ANOVA table for individual site (Tim Shea or Nunamara) analysis of the effects of nitrogen (N) and phosphorus (P) fertiliser on the proportion of trees with adult foliage and the height proportion of trees which bore adult foliage in 1996 (3.5 years old) with either tree height or DBH in that year included as a covariate. Refer to Table 5.1 for fertiliser dose rates and time of application. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001.

	Effect	*Covariate*	*N*	*P*	*N*P*
	DF	1	3	2	6
Tim Shea	**Error DF**	21	21	21	21
	Covariate				
% of trees with adult foliage in 1996	DBH in 1996	32.82 ***	1.44 n.s.	3.52 *	1.10 n.s.
% of canopy with adult foliage in 1996	Height in 1996	46.23 ***	0.46 n.s.	6.35 **	1.15 n.s.
% of canopy with adult foliage in 1996	DBH in 1996	37.53 ***	1.15 n.s.	4.83 *	1.08 n.s.

	Effect	*Covariate*	*N*	*P*	*N*P*
	DF	1	3	2	6
Nunamara	**Error DF**	21	21	21	21
	Covariate				
% of trees with adult foliage in 1996	DBH in 1996	8.46 **	2.08 n.s.	4.63 *	1.11 n.s.
% of canopy with adult foliage in 1996	Height in 1996	18.19 ***	1.69 n.s.	7.96 **	3.24 *
% of canopy with adult foliage in 1996	DBH in 1996	10.98 **	0.78 n.s.	6.88 **	2.50 n.s.

Timing experiment

Consistent with the previous results for this experiments, the timing of nitrogen application and its interaction with site, with and without growth traits as covariates, had no significant effect ($p > 0.05$) on the number of trees with adult foliage or the proportion of tree canopy with was adult foliage in 1996, whilst both growth covariates did have a significant positive effect ($p < 0.01$) on the measured traits.

5.3.2 Fertilisation with nitrogen and co-application of paclobutrazol

5.3.2.1 The effect on flower initiation and abundance in juvenile (< 4 years old) trees

Growth

Both paclobutrazol and nitrogen had a significant effect on the growth rate in height and diameter of the young trees (Table 5.7). There were strong, positive effects of nitrogen treatment on growth in both height and diameter. Although equally significant, the efficacy of paclobutrazol appeared to be greater in reducing growth in height more so than in diameter (Figures 5.4 and 5.5). Additionally, there was a slight interaction between nitrogen and paclobutrazol detected in diameter growth rate, along with a complex nitrogen by paclobutrazol by clone interaction (Table 5.7).

Table 5.7 ANOVA table for the effects of clone (Clone) and treatment with nitrogen (N) fertiliser and paclobutrazol (Paclo) on growth and reproductive traits in a trial at Deacons 4. Trees were treated at 2.5 years of age and final measurements were taken 18 months after treatment. Refer to section *5.2.2.1* for description of treatments. F values and levels of significance shown.. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001. [a]The error degrees of freedom given are only for analyses based on plot means (see section *5.2.2.1*). [b]Analysis of the number of umbels per tree was based on individual tree data for only those trees treated with both nitrogen and paclobutrazol (see section *5.2.2.1*), for this trait all effects have the degree of freedom = 1 and the error degrees of freedom = 32.

Effect	*Clone*	*N*	*Paclo*	*N*Paclo*	*N*Clone*	*Paclo*Clone*	*N*Paclo*Clone*
DF	1	2	2	4	2	2	4
Error DF[a]	13	104	104	104	104	104	104
Height when treated	6.37 *	0.13 n.s.	0.23 n.s.	1.62 n.s.	0.60 n.s.	0.16 n.s.	1.94 n.s.
DBH when treated	6.60 *	0.45 n.s.	0.28 n.s.	1.55 n.s.	0.37 n.s.	0.35 n.s.	1.75 n.s.
Height 18 months post-treatment	4.40 n.s.	3.72 *	20.68 ***	1.20 n.s.	0.15 n.s.	0.34 n.s.	0.99 n.s.
DBH 18 months post-treatment	5.59 *	16.67 ***	5.82 **	1.91 n.s.	0.00 n.s.	0.35 n.s.	2.17 n.s.
Increase in height	2.66 n.s.	10.13 ***	44.85 ***	1.11 n.s.	0.00 n.s.	0.32 n.s.	0.53 n.s.
Increase in DBH	3.12 n.s.	48.28 ***	18.42 ***	3.50 *	0.47 n.s.	0.53 n.s.	2.60 *
% of trees with umbels 18 months post-treatment	6.57 *	19.67 ***	9.59 ***	1.31 n.s.	0.74 n.s.	0.27 n.s.	0.25 n.s.
Number of umbels per tree 18 months post-treatment[b]	2.10 n.s.	0.27 n.s.	0.00 n.s.	4.34 *	3.31 n.s.	6.04 *	0.77 n.s.

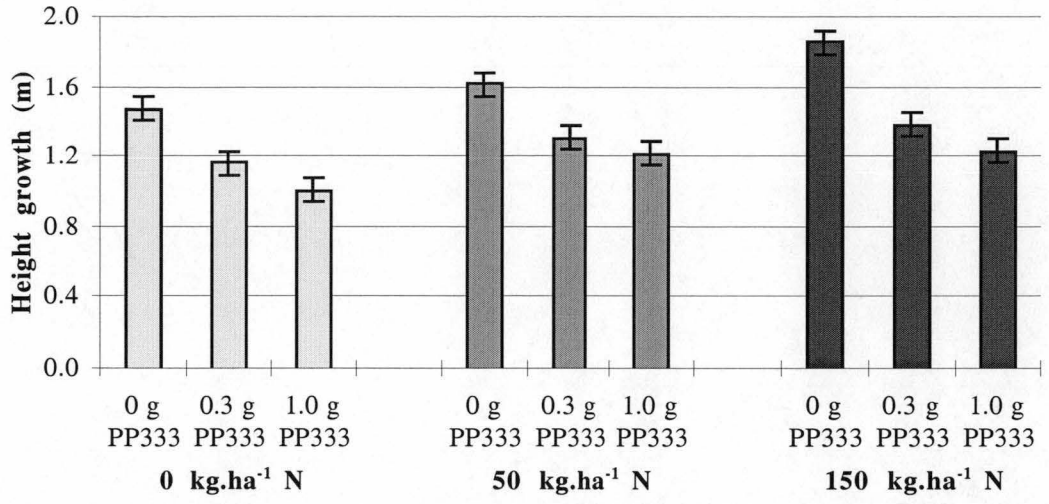

Figure 5.4 Least squares means (±se) of the effect of nitrogen (N) and paclobutrazol (PP333) on the increase in tree height over 18 months (with height when treated included as a covariate) when applied to 2.5 year old trees. The dose rate of paclobutrazol (0, 0.3 or 1.0 g) represents the number of grams applied per centimetre of stem circumference measured at a height of 15 cm and delivered as a collar drench whilst the nitrogen was applied at the specified rates (0, 50 or 150 $kg.ha^{-1}$) by spade slit.

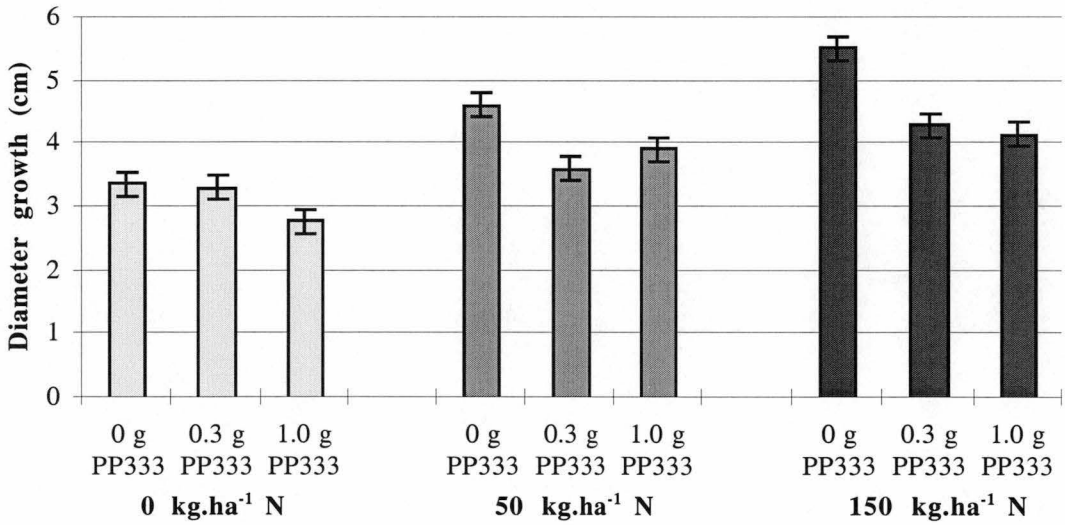

Figure 5.5 Least squares means (±se) of the effect of nitrogen (N) and paclobutrazol (PP333) on the increase in tree diameter (measured at a height of 15 cm) over 18 months (with diameter when treated included as a covariate) when applied to 2.5 year old trees. The dose rate of paclobutrazol (0, 0.3 or 1.0 g) represents the number of grams applied per centimetre of stem circumference measured at a height of 15 cm and delivered as a collar drench whilst the nitrogen was applied at the specified rates (0, 50 or 150 $kg.ha^{-1}$) by spade slit.

Tree diameter at the commencement of treatment (initial diameter), had a positive (r = 0.2958) and significant effect on diameter growth rate as a covariate (Table 5.8). Additionally, its inclusion as a covariate increased the level of significance of the nitrogen by paclobutrazol interaction effect whilst it removed the significance of the three-way interaction (Table 5.8). The low rate of paclobutrazol was virtually ineffective in reducing diameter growth rate on its own whilst it had a substantial inhibitory effect when nitrogen was applied (Figure 5.5).

Tree height at the commencement of treatment (initial height), had a strong, positive (r = 0.4949) and significant effect on height growth rate as a covariate (Table 5.8). Additionally, there were significant effects on height growth of clone and a clone by initial height interaction (Table 5.8). Trees of clone 1 were initially significantly larger in both height and diameter than clone 2 (Table 5.7), with a mean height and diameter for clone 1 of 149.5 cm and 36.1 cm respectively, compared to 130.2 cm and 30.7 cm respectively for clone 2. However, after treatment there was no significant difference between the clones in height whilst there remained a slightly significant difference in diameter (Table 5.7).

Table 5.8 ANOVA table for the effects of clone (clone) and treatment with nitrogen (N) fertiliser and paclobutrazol (Paclo) on growth and reproductive traits with tree size traits (height and DBH) included as a covariate in a trial at Deacons 4. Trees were treated at 2.5 years of age and final measurements were taken 18 months after treatment. Refer to section *5.2.2.1* for description of treatments. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant ($p > 0.05$), * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. [a]The error degrees of freedom given are only for analyses based on plot means. [b]Analysis of the number of umbels per tree was based on individual tree data for only those trees treated with both nitrogen and paclobutrazol, for this trait all effects have the degree of freedom = 1 and the error degrees of freedom for the main effects = 32 whilst covariate effects = 47.

	Effect	*Covariate*	*Covariate* Clone*	*Clone*	*N*	*Paclo*	*N*Paclo*	*N*Clone*	*Paclo *Clone*	*N*Paclo* Clone*
	DF	1	1	1	2	2	4	2	2	4
	Error DF[a]	102	102	13	102	102	102	102	102	102
	Covariate									
Increase in height	Height when treated	44.46 ***	5.90 *	6.60 *	14.61 ***	51.79 **	0.89 n.s.	0.28 n.s.	0.21 n.s.	0.96 n.s.
Increase in DBH	DBH when treated	21.56 ***	2.81 n.s.	3.70 n.s.	54.26 ***	21.58 ***	3.74 **	0.77 n.s.	0.62 n.s.	2.19 n.s.
% of trees with umbels 18 months post-treatment	Height when treated	52.84 ***	0.11 n.s.	0.34 n.s.	25.91 ***	13.43 ***	2.05 n.s.	0.26 n.s.	0.63 n.s.	0.11 n.s.
% of trees with umbels 18 months post-treatment	DBH when treated	38.55 ***	0.02 n.s.	0.12 n.s.	25.82 ***	11.39 ***	2.28 n.s.	1.39 n.s.	0.61 n.s.	0.10 n.s.
Number of umbels per tree 18 months post-treatment[b]	Height 18 months post-treatment	10.54 **	0.74 n.s.	0.64 n.s.	2.48 n.s.	0.12 n.s.	1.21 n.s.	2.09 n.s.	4.72 *	0.12 n.s.
Number of umbels per tree 18 months post-treatment[b]	DBH 18 months post-treatment	8.44 **	1.47 n.s.	1.34 n.s.	1.15 n.s.	1.14 n.s.	1.42 n.s.	3.08 n.s.	3.82 n.s.	0.02 n.s.

Reproduction

Nitrogen, paclobutrazol and clone significantly affected the number of trees with umbels in the first reproductive season (Table 5.7). Whilst initial tree height and diameter were also very significant (positive) in affecting this trait, they could not account for the treatment effects and only the clone effect was removed (Table 5.8). It was the combination of the highest dose of nitrogen and either dose rate of paclobutrazol which induced the highest number of trees to produce umbels (Figure 5.6). Overall, the best results were obtained by combining both nitrogen and paclobutrazol whereas little response was obtained in using either treatment alone (Figure 5.6).

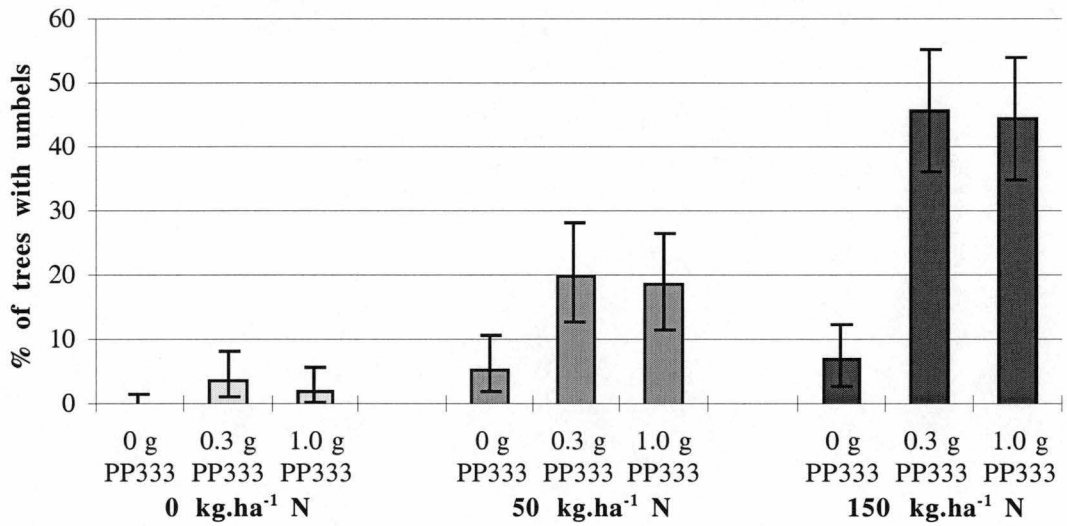

Figure 5.6 Least squares means (±se) of the effect of nitrogen (N) and paclobutrazol (PP333) on the proportion of 4 year old trees with umbels, 18 months after treatment. The dose rate of paclobutrazol (0, 0.3 or 1.0 g) represents the number of grams applied per centimetre of stem circumference measured at a height of 15 cm and delivered as a collar drench whilst the nitrogen was applied at the specified rates (0, 50 or 150 $kg.ha^{-1}$) by spade slit.

Final tree size, both height and diameter, were the most significant effects on the number of umbels per tree treated with nitrogen and paclobutrazol (Table 5.8). The number of umbels per tree was positively correlated with both final tree height (r = 0.3710) and diameter (r = 0.3133). There were no significant main effects of either paclobutrazol or nitrogen whilst there were slightly significant nitrogen by paclobutrazol and paclobutrazol by clone interactions (Table 5.7). However, both of these interactions where no longer significant after accounting for final tree diameter (Table 5.8). These results suggests the differences between the clones in their production of umbels in response to paclobutrazol is basically due to differences in growth rate and not a clonal difference in flowering response *per se*.

5.3.2.2 The effect on flower initiation and abundance in mature (> 4 years old) trees.

Growth

Paclobutrazol and soil type significantly affected diameter and height increments whilst nitrogen only significantly affected diameter growth rate (Table 5.9). The effect of paclobutrazol on diameter and height increment was negative whilst nitrogen generally had a positive effect (Figures 5.7 and 5.8). The mean height and diameter increase on the grey soil, 285.8 cm and 2.4 cm respectively, was greater than on the red soil, 244.6 cm and 2.1 cm respectively. However, there were no significant differences between soil types in the initial and final measurements for either trait (Table 5.9) and the effect of soil on the rate of height and diameter growth was no longer significant after accounting for either initial height or diameter (Table 5.10). Initial mean tree height and diameter on the red soil was 734.3 cm and 8.3 respectively, compared to 699.9 cm and 7.9 cm respectively on the grey soil. Whilst final mean tree height and diameter on the red soil was 977.2 cm and 10.4 cm respectively, compared to 985.9 cm and 10.4 cm respectively, on the grey soil.

Table 5.9 ANOVA table for the effects of soil type (soil) and treatment with nitrogen (N) fertiliser and paclobutrazol (Paclo) on growth and reproductive traits in a trial at Nunamara. Trees were treated at 4 years of age and final measurements were taken 18 months after treatment. Refer to section *5.2.2.2* for description of treatments. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001. [a]The error degrees of freedom given are only for analyses based on plot means. [b]Analysis of the number of umbels per tree was based on individual tree data for only those trees with umbels, for this trait the error degrees of freedom = 44.

Effect	*Soil*	*N*	*Paclo*	*N*Paclo*	*N*Soil*	*Paclo*Soil*	*N*Paclo*Soil*
DF	1	1	1	1	1	1	1
Error DF[a]	12	36	36	36	36	36	36
Height when treated	1.52 n.s.	1.39 n.s.	1.10 n.s.	0.03 n.s.	0.14 n.s.	3.18 n.s.	0.47 n.s.
DBH when treated	0.70 n.s.	0.32 n.s.	0.00 n.s.	0.77 n.s.	1.84 n.s.	1.17 n.s.	0.88 n.s.
Height 18 months post-treatment	0.07 n.s.	1.46 n.s.	13.23 ***	0.92 n.s.	0.26 n.s.	2.52 n.s.	0.00 n.s.
DBH 18 months post-treatment	0.01 n.s.	3.25 n.s.	10.02 **	0.06 n.s.	1.71 n.s.	1.07 n.s.	0.88 n.s.
Increase in height	10.43 **	1.20 n.s.	52.67 ***	3.07 n.s.	0.49 n.s.	0.82 n.s.	0.02 n.s.
Increase in DBH	5.31 *	34.54 ***	48.98 ***	0.70 n.s.	0.47 n.s.	0.02 n.s.	0.00 n.s.
% of trees with umbels 18 months post-treatment	0.65 n.s.	8.17 **	24.18 ***	0.10 n.s.	2.21 n.s.	3.78 n.s.	1.11 n.s.
Number of umbels per tree 18 months post-treatment[b]	0.54 n.s.	0.76 n.s.	37.48 ***	0.14 n.s.	0.12 n.s.	0.83 n.s.	1.86 n.s.

Table 5.10 ANOVA table for the effects of soil (soil) and treatment with nitrogen (N) fertiliser and paclobutrazol (Paclo) on growth and reproductive traits with tree size traits (height and DBH) included as a covariate in a trial at Nunamara. Trees were treated at 4 years of age and final measurements were taken 18 months after treatment. Refer to section *5.2.2.2* for description of treatments. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001. [a]The error degrees of freedom given are only for analyses based on plot means. [b]Analysis of the number of umbels per tree was based on individual tree data and for this trait the error degrees of freedom for the main effects = 44 whilst covaraite effects = 96.

	Effect	*Covariate*	*Covariate* Soil*	*Soil*	*N*	*Paclo*	*N*Paclo*	*N*Soil*	*Paclo*Soil*	*N*Paclo* Soil*
	DF	1	1	1	1	1	1	1	1	1
	Error DF[a]	34	34	12	34	34	34	34	34	34
	Covariate									
Increase in height	Height when treated	8.02 **	0.48 n.s.	0.15 n.s.	0.65 n.s.	64.57 ***	3.50 n.s.	0.52 n.s.	0.08 n.s.	0.09 n.s.
Increase in DBH	DBH when treated	1.62 n.s.	0.09 n.s.	0.00 n.s.	35.25 ***	49.17 ***	1.05 n.s.	0.22 n.s.	0.00 n.s.	0.03 n.s.
% of trees with umbels 18 months post-treatment	Height when treated	5.85 *	0.02 n.s.	0.02 n.s.	7.55 **	23.05 ***	0.04 n.s.	2.91 n.s.	6.61 *	2.05 n.s.
% of trees with umbels 18 months post-treatment	DBH when treated	9.73 **	0.70 n.s.	0.74 n.s.	12.11 **	28.66 ***	0.44 n.s.	4.72 *	6.49 *	0.66 n.s.
Number of umbels per tree 18 months post-treatment[b]	Height 18 months post-treatment	32.48 ***	0.01 n.s.	0.06 n.s.	0.49 n.s.	76.60 ***	0.00 n.s.	0.00 n.s.	2.58 n.s.	1.62 n.s.
Number of umbels per tree 18 months post-treatment[b]	DBH 18 months post-treatment	59.82 ***	0.01 n.s.	0.09 n.s.	0.05 n.s.	86.50 ***	0.14 n.s.	0.16 n.s.	1.57 n.s.	0.43 n.s.

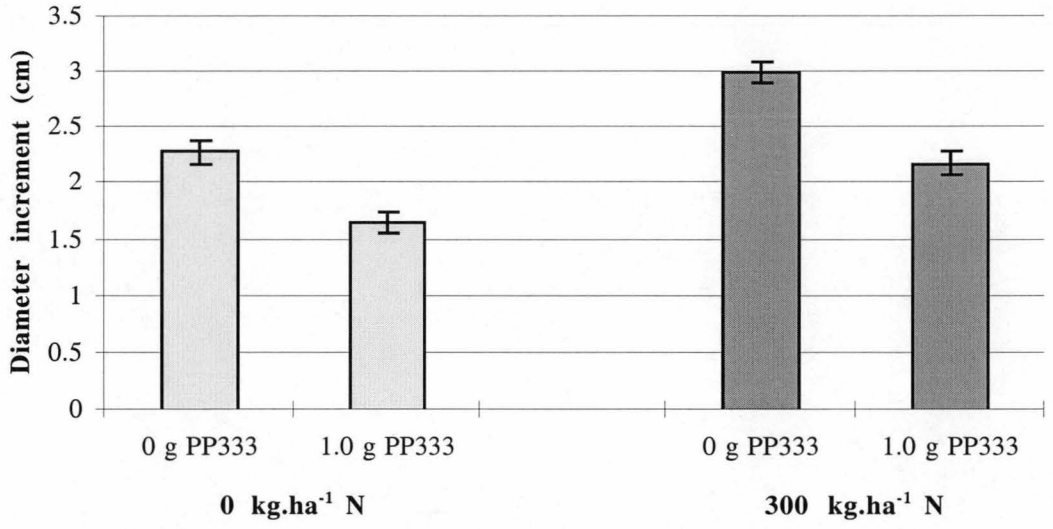

Figure 5.7 Least squares means (±se) of the effect of nitrogen (N) and paclobutrazol (PP333) on the increase in tree diameter over 18 months when applied to 4 year old trees. The dose rate of paclobutrazol (0 or 1.0 g) represents the number of grams applied per centimetre of stem circumference measured at breast height and delivered as a collar drench whilst the nitrogen was applied at the specified rates (0 or 300 $kg.ha^{-1}$) by hand broadcast.

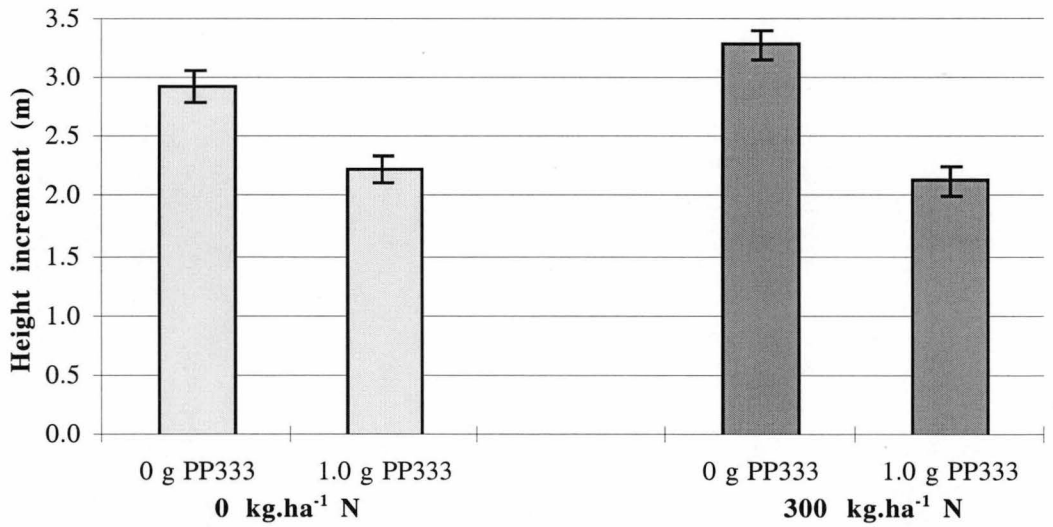

Figure 5.8 Least squares means (±se) of the effect of nitrogen (N) and paclobutrazol (PP333) on the increase in tree height over 18 months when applied to 4 year old trees. The dose rate of paclobutrazol (0 or 1.0 g) represents the number of grams applied per centimetre of stem circumference measured at breast height and delivered as a collar drench whilst the nitrogen was applied at the specified rates (0 or 300 $kg.ha^{-1}$) by hand broadcast.

Reproduction

Both paclobutrazol and nitrogen significantly affected the number of trees with umbels in the season following treatment, with paclobutrazol having the most significant effect (Table 5.9). Whilst tree size close to the time of initiation significantly increased the proportion of trees with umbels, this did not change the significance of the treatment effect (Table 5.10). After accounting for initial tree size, significant differences between the soil types in the percentage of trees with umbels in response to the treatments were detected (Table 5.10). Nitrogen only had a significant effect of increasing the percentage of trees with umbels on the grey soil type, whilst the effect of paclobutrazol was highly significant on the red soil type but had little or no significant effect (depending on tree size trait used as a covariate) on the grey soil type (Table 5.11). The effect of these complex interactions meant that the maximum number of trees with flowers on both soil types could only be achieved with the combined treatments on nitrogen and paclobutrazol (Figure 5.9).

The number of umbels per tree was significantly affected only by paclobutrazol both with and without compensation for tree size which was also significant (Tables 5.9 and 5.10). Treatment with paclobutrazol resulted in an 8 to 9 fold increase in the number of umbels per tree (Figure 5.10).

Table 5.11 ANOVA table for individual soil type analysis (Red and Grey) at Nunamara of the effects of treatment with nitrogen (N) fertiliser and paclobutrazol (Paclo), on the proportion of trees with umbels in 1998 (5.5 years old and 18 months after treatment) with either tree height or DBH when treated included as a covariate. Refer to section *5.2.2.2* for fertiliser and paclobutrazol dose rates. F values and levels of significance shown. Key: DF = degrees of freedom, n.s. = not significant (p >0.05), * = p <0.05, ** = p <0.01, *** p <0.001.

	Effect	*Covariate*	*N*	*Paclo*	*N*Paclo*
	DF	1	1	1	1
Red Soil	**Error DF**	17	17	17	17
	Covariate				
% of trees with umbels	Height when	7.64	1.08	55.43	2.53
18 months post-treatment	treated	*	n.s.	***	n.s.
% of trees with umbels	DBH when	4.66	1.42	49.67	1.84
18 months post-treatment	treated	*	n.s.	***	n.s.

	Effect	*Covariate*	*N*	*Paclo*	*N*Paclo*
	DF	1	1	1	1
Grey Soil	**Error DF**	17	17	17	17
	Covariate				
% of trees with umbels	Height when	0.85	7.67	2.86	0.84
18 months post-treatment	treated	n.s.	*	n.s.	n.s.
% of trees with umbels	DBH when	6.17	14.31	4.67	0.14
18 months post-treatment	treated	*	**	*	n.s.

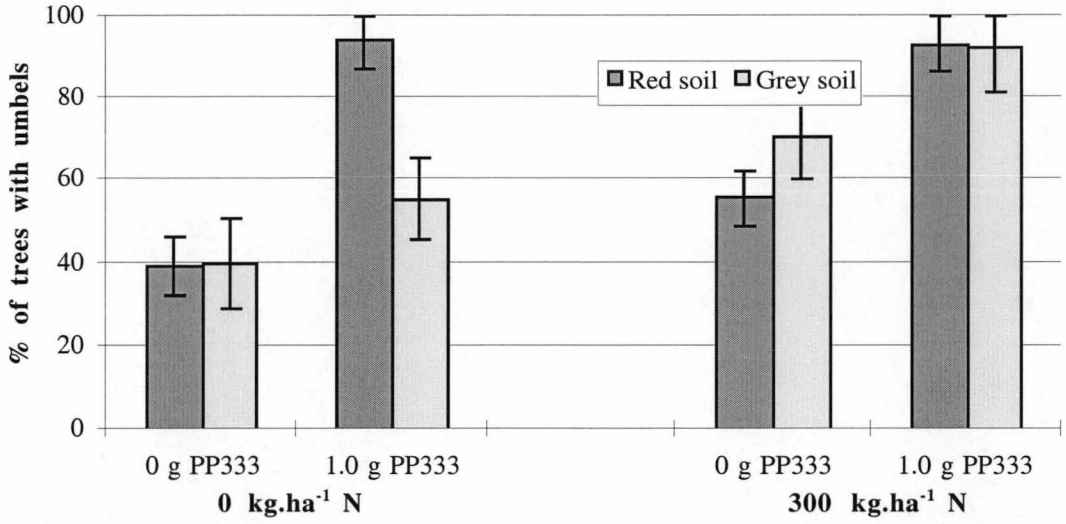

Figure 5.9 Least squares means (±se) of the effect of nitrogen (N) and paclobutrazol (PP333) on the proportion of 5.5 year old trees with umbels on two different soil types, assessed 18 months after treatment and with stem diameter when treated included as a covariate. The dose rate of paclobutrazol (0 or 1.0 g) represents the number of grams applied per centimetre of stem circumference measured at breast height and delivered as a collar drench whilst the nitrogen was applied at the specified rates (0 or 300 kg.ha^{-1}) by hand broadcast.

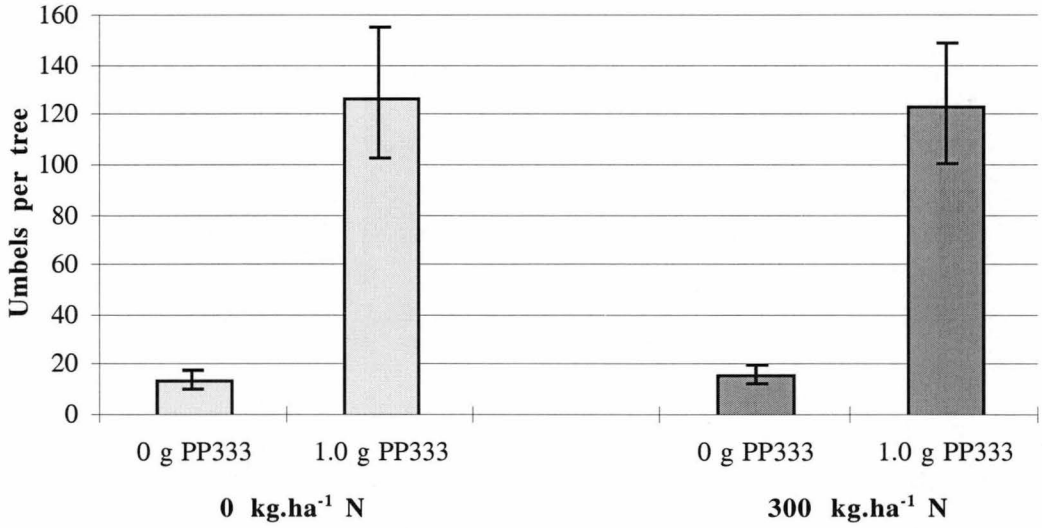

Figure 5.10 Least squares means (±se) of the effect of nitrogen (N) and paclobutrazol (PP333) on the number of umbels per reproductive tree at 5.5 years of age, assessed 18 months after treatment. The dose rate of paclobutrazol (0 or 1.0 g) represents the number of grams applied per centimetre of stem circumference measured at breast height and delivered as a collar drench whilst the nitrogen was applied at the specified rates (0 or 300 kg.ha^{-1}) by hand broadcast.

5.4 DISCUSSION

The effectiveness and reliability of paclobutrazol in promoting precocious flowering in *E. nitens* can be improved through the application of nitrogen fertiliser, and vice versa. The complementary mechanisms may result from either exogenous (eg. geochemical) or endogenous (eg. biochemical) activity or a combination of both. Although there was no evidence of a synergistic response between the hormonal and cultural treatments, suggested by Bonnet-Masimbert and Webber (1995), the effect on precocious flowering when applied to 2.5 year old trees (Deacons 4) appear to be more than additive. In gymnosperms, manipulation of nitrogen supply and GA levels significantly changed the amino acid content of shoots preceding flower initiation (Daoudi *et al.* 1994). In the shoot apex of *E. nitens* there is a strong relationship between GA_1 levels and flower abundance but the relationship is only shown after a cold period equivalent to a full winter (Moncur and Hasan 1994). It would be of interest to examine the effect of nitrogen on the shoot apex biochemistry and if there are any correlations with GA levels or the obligate cold requirement.

Precocious reproductive development was most commonly associated with greater tree size in all experiments. Increases in both height and DBH, significantly increased the occurrence of first flowering (precocity) and the abundance of umbels in trees from 3.5 to 5.5 years old. A similar relationship between tree size and flowering precocity was found in 4 year old *E. regnans* (Cameron and Kube 1983) and 4 year old *E. globulus* (Chambers *et al.* 1997), supporting the suggestion that plant size or growth rate are significant factors in determining when first flowering will occur (Hackett 1985).

However, the importance of tree size on precocity diminishes in significance once a minimum threshold size has been reached. For example, the significance of the effect of size on precocity in the Tim Shea and Nunamara fertiliser trials reduced from 1995 to 1996. Furthermore, in the Nunamara nitrogen and paclobutrazol experiment, planted at the same time as the fertiliser experiment, the significance of the tree size effect in 1997 had reduced further. However, there still may be a strong genetic component to precocity at an individual level (Jordan *et al.* 1999).

Increased tree size significantly increased the abundance of umbels on reproductive trees at 4.5 and 5.5 years of age. This relationship had been reported in a number of young (< 10 years old), orchard grown, forest tree species including lodgepole (Wheeler *et al.* 1982), aleppo (Matziris 1997) and radiata (Griffin *et al.* 1984) pine and *E. regnans* (Cameron and Kube 1983). However, this relationship may occur only under certain growth conditions and may not persist as trees age. Griffin (1980) found flowering intensity to be independent of tree size in a mature (trees >15 years old) natural stand of *E. regnans* and no significant effects of tree size were found on flowering abundance in 14 to 15 year old plantations of *E. nitens* along an altitudinal transect (Chapter 2). Indeed as Wheeler *et al.* (1982) found in comparing accelerated seedlings and ramets of lodgepole pine, the number of potential flowering sites on a tree (i.e. tree size) was not the most critical factor in determining flowering abundance.

Accelerating growth through the application of fertiliser, specifically nitrogen, benefits both precocious and abundant flowering. This was shown in trees up to 5.5 year old and may have potential benefits in older trees as well. The application of nitrogen fertilisers has been shown to significantly improve growth rates in *E. globulus* to 9.5 years of age and *E. nitens* to at least seven years of age (Neilsen 1996, Turnbull *et al.* 1997) with the

growth rate response of *E. nitens* to nitrogen increasing to at least 5.5 years of age (R. Cromer pers. comm.).

The positive effects of nitrogen on flowering are two fold. Whilst nitrogen enhanced growth rate was shown to be a major contributor to improved flowering, growth rate alone could not account for all the increases observed in these experiments. Nitrogen significantly increased both the percentage of trees with umbels (Tables 5.8 and 5.10) and umbel abundance (Table 5.4) independently of growth. Fertilisation of trees with nitrogen has been a regular practice in orchards of horticultural and forest species for many years as it is known to increase flower production by more than could be explained by growth rate (Jackson and Sweet 1972, Sedgley and Griffin 1989, Owens 1991). Although Cameron and Kube (1983) did find fertiliser increased the occurrence and abundance of flowering in *E. regnans*, they believed it to be merely a function of tree size. The results here demonstrates there is indeed more to the effect of nitrogen on flowering in eucalypts than is mediated by tree size and present another tool which could be used to explore the mechanism which controls flowering in this genus.

There were some across site inconsistencies where nitrogen affected the occurrence of flowering at Deacons 4 (Table 5.7) and at Nunamara (Table 5.9) in the nitrogen by paclobutrazol experiments whilst the abundance of flowering was affected at Tim Shea (Table 5.4) in the nitrogen by phosphorus experiment. Factors which may have contributed to this would include relative dose rates and mode of application, soil conditions and tree age. The significant effects of the relatively low nitrogen dose at Deacons 4 may be a function of the method of application as spade slit application is considered to be a more effective means than surface application, particularly for smaller trees (Herbert 1996). As nitrogen affected tree height in all experiments except in the nitrogen by paclobutrazol experiment at Nunamara (Table 5.9) this indicates the dose rate

used in this experiment was conservative and as a consequence, may be responsible for its lack of effectiveness in promoting abundant flowering. The modest application of nitrogen to the older trees at Nunamara did reveal a sensitivity to soil conditions of the flowering response. Indeed, the variation between Tim Shea and Nunamara in the nitrogen by phosphorus experiment may be due to the higher levels of naturally available nitrogen at Tim Shea (Wang *et al.* 1998) which may have also be responsible for greater growth in diameter at Tim Shea in 1997.

The evidence suggests as trees age the sensitivity to manipulation of both occurrence and abundance of flowering with nitrogen changes. The younger trees at Tim Shea responded significantly in the abundance of flowering but not in the occurrence of flowering whilst the trees at Nunamara treated at a later age in the nitrogen by paclobutrazol experiment responded significantly in the occurrence but not in abundance of flowering. This suggests there are potentially different though not mutually exclusive qualitative and quantitative mechanisms controlling flowering (Bernier *et al.* 1981).

Phosphorus had a significant affect on the timing of vegetative phase change whilst having no affect on flowering. There was a clear trend with increasing phosphorus supply, the earlier the trees switched from juvenile to adult foliage. This was independent of tree size and evident in both the qualitative and quantitative measurements of the trait. The mechanism which controls when eucalypts will change from juvenile to adult leaves is unclear, though it is thought to be related to the physiological rather than chronological age of the apical meristem (Wiltshire and Reid 1992). Vegetative phase change traits are highly heritable (Jordan *et al.* 1999) and the genetic by environment interaction complex (Jordan *et al.* in press). In this experiment, the trees are of random genetic make-up, the same chronological age and the phosphorus treated trees were not significantly different in size which suggest their physiological age was reasonably uniform, the effect of

phosphorus was expressed at two different sites. These markedly different responses in the development of reproductive and vegetative maturity to the fertiliser treatments is further evidence of a decoupling between the two physiological processes in *E. nitens* as observed in *E. globulus* (Jordan *et al.* 1999) and *E. tenuiramis* (Wiltshire *et al.* 1998).

The early transition to adult foliage shown may be indicative of an adaptive response to low phosphorus soils. Australian soils are relatively low in available phosphorus which can limit growth (McLaughlin 1996). Juvenile leaves of *E. globulus* have been shown to have a higher relative proportion of phosphorus than that of the adult form (Judd *et al.* 1996). When there is a high level of phosphorus in eucalypt leaves, a significant proportion is of an easily mobilised form and translocated as needed (Grove *et al.* 1996). Young *E. nitens* trees (< 3 years old) have shown a capacity to opportunistically collect and store phosphorus in leaves for later use (Misra *et al.* 1998). It is therefore possible that a significant part of the high level of phosphorus in juvenile leaves of *E. nitens* is in a form that can be easily remobilised from the juvenile leaves to other parts of the tree prior to leaf abscission if supply is limited. Furthermore, if phosphorus supply is not limiting, this resource storage feature of the juvenile leaves may be redundant. Juvenile leaves may therefore not be produced by the tree for as long resulting in early transition to the adult leaf form.

The poor flowering response to paclobutrazol when applied alone to vegetatively juvenile *E. nitens* trees at Deacons 4 is typical of this species (Griffin *et al.* 1993, Moncur 1998 Chapter 4) despite the evidence of decoupling between vegetative and reproductive maturity. This is in strong contrast to the effectiveness of paclobutrazol seen in the vegetatively mature trees at Nunamara and observed by other authors (Griffin *et al.* 1993, Moncur *et al.* 1994) and juveniles of the closely related *E. globulus* (Hasan and Reid

1995). Indeed the application of nitrogen fertiliser would appear to be as, if not more, effective in inducing precocious flowering in young *E. nitens*.

Environmental conditions can alter the effectiveness of paclobutrazol (Leaver *et al.* 1982), and application via soil drench is no exception (Moncur *et al.* 1994, Swain and Chiappero 1998) and was seen in the experiment at Nunamara when applied across two soil types. Although it is not clear what qualities of the soil altered the effectiveness of paclobutrazol, there was a substantial benefit of applying nitrogen.

The ability to control growth is a further benefit of the combined nitrogen and paclobutrazol treatment. With the inherent difficulties of crown management when working with forest tree species (Eldridge *et al.* 1993), the application of luxuriant levels of fertiliser could be seen to magnify the problem. However, regardless of nitrogen dose, in all treatments with paclobutrazol, the mean height increase of trees was less than that of the controls. In contrast, the mean increase in tree diameter in response to nitrogen at Deacons 4 was greater than that of the controls, regardless of paclobutrazol dose. Fertilisation with high levels of nitrogen has been shown to produce 'stockier' trees with larger branches and a greater occurrence of multiple leaders (Neilsen 1996). In an orchard this may actually be advantageous as it would promote a more bushy tree with more potential flowering sites close to the ground. Additionally, thicker trunks and branches may benefit tree health in the case of a concomitant increase in resource demand bought to bear by the heavy flower and capsule crop induced by the treatments.

The dose rate of paclobutrazol which provided the best levels of both flower initiation and growth control was 1.0 g per centimetre of stem circumference. This is largely based on the comparison with the lower 0.3 g per centimetre of stem circumference at Deacons 4 and assumes a good supply of nitrogen. As a guide to what level of nitrogen supply might

be necessary for optimum flower production, the strong associations with tree size suggest the methods available for determining the best rate of application for growth (Judd *et al.* 1996, Misra *et al.* 1998) would be suitable. As juvenile *E. nitens* trees tend to have square shaped stems which are difficult to measure for diameter, dose rates for paclobutrazol should be calculated using basal diameter (15 cm above soil level) until the stem at breast height (130 cm above soil level) has developed its circular shape. If strong control of growth rate was not of a primary concern, a dose rate of paclobutrazol of 0.3 g per centimetre of stem circumference would still be as beneficial to flower production as the higher rate and would be cheaper to implement. However, to optimise and explore the full potential of the combined treatment it would be necessary to carry out dedicated experiments. Indeed, exploring the complementary activity of nitrogen and paclobutrazol in the promotion of flowering may provide a tool to further study the mechanism of flowering in this species.

Further issues which would need to be explored would include the persistence of the effects from one year to the next and the requirements to maintain optimum production, the effects on capsule and seed production and on seed quality.

In conclusion, growth rate has a major impact on flowering with increased size generally resulting in earlier and more abundant flowering. Many of the flowering responses to fertiliser are mediated through the effect on growth rather than on flowering *per se*. However, nitrogen does affect flowering precocity and abundance in a way which could not be accounted for by tree size. The reliability of either nitrogen or paclobutrazol as a flowering promoter can vary with tree age and soil type. A more reliable response can be achieved through the combined application of nitrogen and paclobutrazol. In young trees (< 4 years old), flowering precocity was markedly enhanced by the combined application of paclobutrazol and nitrogen. In older trees, the response to paclobutrazol was more

uniform across soil types after nitrogen application. While phosphorus had no significant effect on tree growth or flowering an unexpected effect on vegetative maturity was detected. As supply of phosphorus to the tree increased, the earlier the transition to adult foliage occurred.

Chapter 6

Testing single visit pollination procedures for *Eucalyptus globulus* and *E. nitens*

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6.1 INTRODUCTION

In *Eucalyptus globulus* and *E. nitens*, controlled pollination (CP) is performed extensively for breeding and research purposes (eg. Tibbits 1989, Tibbits 1997, Hardner *et al.* 1996). Both species develop simple axillary inflorescences. *E. globulus* ssp. *globulus* has an umbel usually comprising a single, relatively large flower approximately 15 mm in diameter whilst *E. nitens* has an umbel of typically 7 flowers, each approximately 4 mm in diameter. The flowers of both species are bisexual with the style surrounded by the anthers, and natural pollination is mediated by foraging animals and insects (Griffin 1982b, Hingston and Potts 1998). Whilst protandry provides some barrier to fertilisation of a flower by its own pollen (autogamous self pollination), pollination from other flowers on the same tree (geitonogamous self pollination) occurs readily (Hardner *et al.* 1996, Tibbits 1989). These floral characteristics make it necessary for a number of steps to be taken during controlled pollination to ensure that a flower is only fertilised by the applied pollen.

The current, widely accepted technique for controlled pollinating eucalypt flowers requires three visits to the female tree for: (1) emasculation at operculum lift to prevent self fertilisation and isolation of the flower with a bag to exclude foreign pollen; (2) pollination at stigma receptivity, 3 to 28 days after operculum lift depending on species and environment (Oddie and McComb 1998); and (3) de-bagging after fertilisation, typically 3 to 4 weeks after pollination (Moncur 1995, Tibbits 1986). This process can be time consuming and costly, particularly if it needs to be performed at great distance from the normal base of operations and/or requires specialist equipment such as hoists.

Three modifications to the standard controlled pollination method have been previously employed which have the potential to reduce the number of operational visits to the female tree from three to one. One method, tested on *E. grandis* (Hodgson 1976), *E. gunnii* (Cauvin 1983) and *E. camaldulensis* (Oddie and McComb 1998), demonstrated that although reduced, some seed set occurs when the pollen is applied to the dry, non-receptive stigma, at anthesis. In this context, anthesis is defined as the time at which the operculum begins to lift (Tibbits 1989). Eucalypt pollen is relatively robust and can remain viable for days to weeks in ambient conditions if kept dry (Pryor 1976). Such conditions would tend to prevail in pollination bags and the dormant pollen would remain on the styles, then germinate once the stigma became receptive.

The second modification to the standard method employed with *E. globulus* involves excluding pollen by fitting a small piece of plastic tube, sealed at one end, over the style following emasculation (Barbour 1997). Currently, the tube is removed to allow for the application of pollen at peak stigma receptiveness, then re-fitted. After fertilisation, the stigma abscisses taking the tube with it. Use of this method to prevent contamination would mean that all available flowers could be used for crossing- unlike bag isolation where flowers that are pre- or post-anthesis, and likely to be enclosed in a bag with the

crosses, must be removed to prevent contamination. Either of these two methods reduced the number of visits from three to two and could potentially be combined to reduce the operation to a single visit.

A third modification reported for *E. gunnii*, involved cutting, splitting or abrading the style following emasculation and applying pollen immediately to the fresh wound surface of the remaining style (Cauvin 1988). These treatments, particularly style cutting, produced greater numbers of seeds than applying the pollen to the untreated dry stigma. It was felt that isolation of the flowers was unnecessary as the wound would rapidly oxidise and form a protective barrier against extraneous pollen (Cauvin 1988).

Before operationally implementing a single visit pollination method- which uses pollination at anthesis with or without partial removal of the style, and with or without the tube isolation method- the reliability of the system needed to be tested. This chapter reports on the success of different combinations of the treatments described above on two commercially important and floristically different species *E. globulus* and *E. nitens* - with the aim of developing a practical single visit pollination method with potential application to a broad range of eucalypt species.

6.2 METHODS AND MATERIALS

6.2.1 Effect of style treatments in E. globulus and E. nitens

6.2.1.1 Genetic material

Three *E. globulus* ssp. *globulus* trees on the Tinderbox Peninsula south of Hobart, Tasmania, were selected as females for crossing in November 1996. Selection was based on previous capsule retention, and the abundance and number of accessible flowers for hand pollination. These trees were crossed with polymix pollen collected from 14 trees from the same region. The pollen of each tree was mixed in approximately equal proportions after extraction and dry stored at -18°C until required (Potts and Marsden-Smedley 1989). *Eucalyptus nitens* crosses were carried out in January and February 1997 and 1998 in a seed orchard at Bream Creek in south-eastern Tasmania. Six trees were selected as females in 1997 whilst seven trees were selected in 1998, one of which was also used in the previous year. Selection was done as previously described. The crosses were performed with a 15 tree polymix pollen collected in January 1997 from the same orchard, but excluding the female trees used for pollination trees, and stored as previously described. Prior to use, pollen samples from each species were tested to ensure viability as described in Gore *et al.* (1990).

6.2.1.2 Crossing methods

In both species, five different pollination treatments (1-5) were tested in the 1996/97 season and compared to the control untreated open-pollinated flowers (treatment 9), with

three additional treatments (6-8) tested only on *E. nitens* in 1998. These treatments are summarised in Table 6.1. The treatments aimed at comparing the success of applying pollen immediately after emasculation to the dry stigma (treatment 2), or abraded stigma (treatments 6 and 7), or to the cut style (treatments 3 and 4) with- the normal three visit control (treatment 1), contamination controls (treatments 5 and 8), and open-pollination (non-emasculated flowers; treatment 9). Treatments 1-3 and 6 were bagged to prevent flower contamination whilst treatments 4, 5 and 7-9 were left open to natural pollination. Treatments 5 and 8 were not pollinated by hand and were used to gauge levels of contamination by extraneous pollen of flowers which were not bagged. In all but the open-pollinated treatment (9), the flowers were emasculated at operculum lift with either a scalpel (*E. globulus*) or modified electricians pliers (*E. nitens*; Tibbits 1989). In the cut style treatments (3-5), 10 to 100 percent of the style was removed with a scalpel immediately after emasculation and the approximate percentage of the style remaining recorded as this may affect cross success. In the abraded style treatments (6-8) applied only to *E. nitens* in 1998, the tip of the style was rubbed lightly with a fine emery board. In treatments 3, 4, 6 and 7 the delay from style treatment (i.e. cutting or abrading) to pollen application was kept to a minimum. Where pollen was manually applied, this was done using a match stick which had been dipped in the gelatine capsule containing the dry pollen. The isolation bags used were made of non-woven polyester (Terylene) and held secured to the branches with electricians' wire ties. When flowers were bagged, excessive foliage and opened and unopened flowers unable to be used in the treatments, and likely to be enclosed in the bags, were removed. Bags were removed 28 to 35 days after emasculation.

Table 6.1 Pollination treatments tested on *E. globulus* in 1996 and *E. nitens* in 1997 and 1998

No.	Treatment	Number of flowers treated		
		E. globulus 1996	*E. nitens* 1997	*E. nitens* 1998
1	3 visit control	21	291	252
2	dry stigma pollinated, bagged	12	290	135
3	cut style pollinated, bagged	17	306	196
4	cut style pollinated, unbagged	11	163	146
5	cut style unpollinated, unbagged	11	254	125
6	abraded stigma pollinated, bagged	0	0	180
7	abraded stigma pollinated, unbagged	0	0	134
8	abraded stigma unpollinated, unbagged	0	0	257
9	untreated, open-pollination	13	250	257

6.2.1.3 Seed collection and testing

Approximately twelve months after pollination, random samples of open-pollinated capsules were removed from the female trees. The capsules were dried and the extracted seeds assessed for maturity based on the presence of fully black testa (Moncur 1995). This was repeated as necessary and when all seeds were mature, the treated capsules were collected and stored individually whilst they dried. When the capsule valves were fully opened, the seed was extracted and the number of viable (full seeds), inviable (flat empty seed) or insect damaged seed determined (Hardner and Potts 1995). The healthy viable *E. globulus* seeds were weighed and average individual seed weights calculated.

6.2.1.4 Statistical analysis

In the analysis of the *E. globulus* data, the effect of the various style treatments on the capsules collected per flower treated, number of seeds per capsule and per flower, individual seed weight and proportion of seeds which were inviable were examined based on overall tree proportions or means (in the case of seed weight), for each treatment. A restricted maximum-likelihood (REML) model was fitted using PROC MIXED in SAS (SAS 1992) where pollination treatment was a fixed effect, female tree a random effect and the random interaction was used as the error. The analysis was repeated with the percentage of the remaining style included as a fixed effect. The analysis used the combined results for two trees from which the greatest number of crosses were collected and pollination bag values for just the style trimming treatments.

The effects of style treatment on the number of capsules collected per flower treated, number of seeds per capsule and per flower and proportion of seeds which were inviable in *E. nitens* were assessed using a similar approach to that of *E. globulus*. However, the data for seeds per capsule and per flower was log transformed whilst capsules per flower and proportion of seeds which were inviable was angular transformed prior to fitting the REML model. Specific *a priori* pair-wise contrasts of all pollination treatments were undertaken based on the treatment main effect. The *E. nitens* data was split into two subsets for the analysis. The first sub-set contained the combined results for the 1997 and 1998 seasons excluding the data for the abrasion treatment. In this case, year was included in the analysis as a fixed effect and the female effect was nested within this effect. The second sub-set contained only the data from the 1998 season which included the abrasion treatment.

6.2.2 Testing a single visit pollination protocol

6.2.2.1 Genetic material

Eight mature, native stand *E. globulus* ssp. *globulus* trees were selected for the experiment. Four trees were from north-eastern Tasmania and four from south-eastern Tasmania. These represent two distinct races of *E. globulus* ssp. *globulus* (Dutkowski and Potts 1999) which were being studied because of their markedly different chloroplast haplotypes (Jackson *et al.* 1999). Pollen was collected from all trees in the 1997 season and sub-samples were tested for viability as described in Gore *et al.* (1990).

6.2.2.2 Crossing method

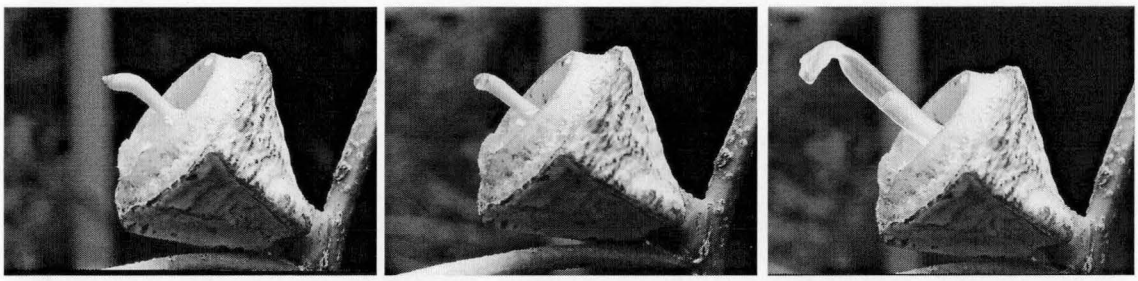
The trees were crossed in the 1997 season to create a full 8 x 8 diallel (where every tree was used both as a female and male, including selfs). This full crossing design was undertaken using the traditional 3 visit method. In addition, on seven of the trees, for each female by male combination, at least one flower was used for each of the single visit treatments (treatments 2-5; Table 6.2).

Table 6.2 Single visit pollination treatments tested on *E. globulus* in 1997

No.	Treatment	Total number of flowers treated
1	3 visit control, bag isolated	516
2	dry stigma pollinated, tube isolated	58
3	cut style pollinated, tube isolated	68
4	dry stigma unpollinated, tube isolated	37
5	cut style unpollinated, tube isolated	34
9	Untreated, open-pollination	.*

*flowers not treated but capsules collected

For single visit pollination (Figure 6.1 a-c), pollen was applied using a matchstick to the dry stigma (treatment 2) and cut style (treatment 3) following emasculation at anthesis whilst their contamination controls (treatments 4 and 5 respectively) were treated similarly except no pollen was applied. When the styles were cut, the stigma and 5-10 % of the style was removed with a scalpel immediately after emasculation and, if pollen was applied, this was done as quickly as possible to the fresh wound. In treatments 2-5, the style was isolated with a small piece of plastic tube (AWG 12 std, UNASCO Pty Ltd). The tube was cut to a minimum length to completely enclose the style, squashed and folded over at one end and pushed on to a snug fit at the base of the style. Occasionally, the tube required the open end to be crimped prior to placement to improve the grip on the style (Barbour 1997). These treatments were compared with the normal three visit technique previously described (Table 6.2).



(a) **(b)** **(c)**

Figure 6.1 Stages in the single visit pollination procedure used for *E. globulus*.

a) Emasculation of the flower by cutting filaments at their base (note: pollen can be applied to the tip of the style at this stage with no further treatment, but this may reduce seed set).

b) The tip (5-10%) of the style is removed and pollen is applied (note: trimming results in the production of a wound exudate on the cut surface which assists pollen adhesion).

c) Isolation of the style with a sealed plastic tube (note: a tight fit is essential to prevent the tube from becoming prematurely dislodged).

6.2.2.3 Seed collection and testing

Approximately 12 months after pollination, open-pollinated capsules were collected from the female trees to test for seed maturity. When the seeds were mature, the crossed capsules and samples of open-pollinated capsules were collected, dried individually and the seeds were assessed for viability, counted and viable seed weighed as previously described.

6.2.2.4 Statistical analysis

The statistical analysis was similar to that used in the previous experiments. Two females (one of each race) were excluded, either due to poor overall capsule set or because single visit treatments (treatments 2-5) were not performed due to a lack of flowers. Self pollinations were also excluded. The effect of the pollination treatments on the capsules collected per flower treated, viable seeds per capsule and per flower, individual seed weight and proportion of seeds which were inviable were examined using tree means for each of the five treatments. As there were no viable seeds produced in the contamination controls (treatments 4 and 5), these treatments were excluded in all analyses except for capsules collected per flower treated. A mixed model was fitted as previously indicated and specific *a priori* pair-wise contrasts of all treatments were undertaken. Pearson correlations between seed weight and number of seeds per capsule were carried out across all treatments and all trees from which capsules were collected.

6.3 RESULTS AND DISCUSSION

6.3.1 Effect of style treatments

The initial testing of the style treatments in *E. globulus* showed that both the application of pollen to either the pre-receptive stigma (treatment 2) or the cut style (treatment 3) at anthesis resulted in seed set which was not significantly ($p > 0.05$) different to the established three visit method (treatment 1) on a per flower and per capsule basis (Table 6.3). In fact, the trend would suggest that the cut style treatment may produce more seeds per flower and per capsule than the traditional three visit pollination technique.

Table 6.3 Least squares means for different pollination treatments applied to *E. globulus* flowers for the proportion of capsules harvested per flower treated, number of viable seed per capsule harvested and per flower treated, the individual seed weight, and the percentage of seeds that were inviable. The significance of the effect of pollination treatment and female tree are indicated (n.s. = not significant). Flowers were emasculated in all treatments except 9.

No.	Treatment	Capsules per flower	Seed per capsule	Seed per flower	Individual seed weight (mg)	% of inviable seeds
1	3 visit control	0.36	16.1	6.4	2.38	8.1
2	dry stigma pollinated, bagged	0.39	18.1	6.3	1.76	15.5
3	cut style pollinated, bagged	0.63	35.8	24.3	2.30	7.7
4	cut style pollinated, unbagged	0.29	32.1	8.4	1.88	3.3
5	cut style unpollinated, unbagged	0.05	4.6	0.1	4.12	50.0
9	untreated, open-pollination	0.50	4.8	2.5	2.34	11.3
	Prob (treatment)	n.s.	n.s.	n.s.	n.s.	n.s.
	Prob (female)	n.s.	n.s.	n.s.	n.s.	n.s.

Previous work where styles of *E. globulus* were cut off at times ranging from anthesis to 6 days after anthesis and directly pollinated, produced no viable seed (K. Badcock and P. Volker, unpublished data). Further work in *E. globulus,* where part of the style was removed at peak receptivity, produced about 50% of the seed obtained following normal controlled pollination (W. Tibbits, unpublished data). In both cases treatments removed from 66 to 100 % of the stigma. Gore *et al.* (1990) found that in *E. globulus*, the transmitting tissue surrounding the stylar canal narrows and converges in the lower half of the style. This would suggest that the chances of applying pollen to the exposed transmitting tissues after trimming of the style would decrease, the greater the length of style that was removed. In the present study no capsules were produced when pollination

was done after 100% of the style was removed, but when the proportion of the style removed was included as a fixed effect in the analysis, no significant effect ($p > 0.05$) on seed production per flower or per capsule was found. However, further investigation of the optimal position to cut the style is warranted.

Cutting the style, particularly near the tip, often resulted in a sticky exudate being formed on the freshly cut surface. This exudate may be derived from the transmitting tissue and be chemically similar to the receptive stigma exudate which improves adhesion of the applied pollen and may contribute to pollen germination and tube growth (Wang *et al.* 1996). The exudate was only visible for a few minutes and appeared to dissipate as the cut surface began to oxidise. Cauvin (1988) found that only 4.2% of seedlings produced from the unisolated cut style treatment of *E. gunnii* originated from contaminating pollen and suggested that the oxidation of the cut style surface may form a barrier to extraneous pollen. The potential for little contamination was also consistent with the present study where very few seeds were produced from the unpollinated, unbagged, cut style treatment (treatment 5; Table 6.3). The number of seeds produced per flower from this open-pollinated cut style treatment was about 4% of the untreated open-pollinated flowers (treatment 9; Table 6.3). This reduced seed set could be attributed to the oxidised style surface forming a partial barrier although the possibility that the emasculated flowers were less attractive to pollinators can not be excluded. The unpollinated, cut style treatment (treatment 5; Table 6.3) produced less than 1% of the seeds per flower than the same treatment where pollen was applied by hand (treatment 4; Table 6.3) suggesting that the contamination level in the pollinated cut style, unbagged treatment is very low. Indeed, under direct competition with the applied pollen, contamination, particularly by self pollen, could be far lower than 1% (see Griffin 1989), and would be at a level acceptable for supplementary mass pollination systems. At this stage, with the success of pollinating

trimmed styles at anthesis in *E. globulus*, the only requirement to achieve a viable single visit controlled pollination method is the application of a compatible isolation method. When the style manipulation and pollination techniques were applied to *E. nitens*, seed production was significantly reduced ($p < 0.05$) compared to the standard three visit control method on a per capsule and per flower basis across both years tested (Tables 6.4 and 6.5). Only pollination of abraded styles at anthesis with bag isolation (treatment 6; Table 6.5) was not significantly different ($p > 0.05$) from the three visit control (treatment 1) on a seed per capsule basis. Whilst not significant, this treatment only produced 48% of the seed per capsule of the three visit control (treatment 1) and seed set was significantly ($p < 0.05$) reduced on a seed per flower basis.

Table 6.4 Least squares means for different pollination treatments applied to *E. nitens* flowers in 1997 and 1998 for the proportion of capsules harvested per flower treated, the number of viable seed per capsule harvested and per flower treated, and percentage of seeds that were inviable. The significance of the effect of pollination treatment, treatment year and female tree within year are indicated (n.s. = not significant). Means have been back-transformed and those with the same letter are not significantly different ($p > 0.05$) based on pair-wise contrasts. Flowers were emasculated in all treatments except 9.

No.	Treatment	Capsules per flower	Seed per capsule	Seed per flower	% of inviable seeds
1	3 visit control	0.26 a	5.49 a	1.46 a	9.1
2	dry stigma pollinated, bagged	0.02 b	1.13 bc	0.10 c	22.0
3	cut style pollinated, bagged	0.06 b	0.65 cd	0.01 c	1.0
4	cut style pollinated, unbagged	0.04 b	0.72 cd	0.07 c	25.0
5	cut style unpollinated, unbagged	0.02 b	0.14 d	0.01 c	5.0
9	untreated, open-pollination	0.41 a	1.82 b	0.71 b	11.5
	Prob (treatment)	<0.001	<0.001	<0.001	n.s.
	Prob (year)	n.s.	n.s.	n.s.	<0.01
	Prob (female)	n.s.	n.s.	n.s.	n.s.

The difference in the success rates of the style treatments between *E. globulus* and *E. nitens* may be due to differences in flower size (see Gore *et al.* 1990). The relatively small flower and style of *E. nitens* is much more delicate and easily damaged, potentially making it less amenable to this method of pollen application. Before advancing to the next step of finding a compatible isolation method for single visit controlled pollination in *E. nitens*, it will be necessary to continue searching for a technique of pollination where seed production is at a level which is commercially competitive with the current three visit method. A better understanding of *E. nitens* flower anatomy and associated pollen behaviour may improve the success rate of pollination at anthesis and lead to the development of an effective single visit pollination method for this and floristically similar eucalypt species.

Table 6.5 Least squares means for different pollination treatments applied to *E. nitens* flowers in 1998 for the proportion of capsules harvested per flower treated, number of viable seed per capsule harvested and per flower treated, and the percentage of seeds that were inviable. The significance of the effect of pollination treatment and female tree are indicated (n.s. = not significant). Means have been back-transformed and those with the same letter are not significantly different ($p > 0.05$) based on pair-wise contrasts. Flowers were emasculated in all treatments except 9.

No.	Treatment	Capsules per flower	Seed per capsule	Seed per flower	% of inviable seeds
1	3 visit control	0.259 a	4.97 a	1.421 a	18.9
2	dry stigma pollinated, bagged	0.007 c	0.23 cd	0.003 c	50.1
3	cut style pollinated, bagged	0.138 ab	0.61 bcd	0.004 c	6.8
4	cut style pollinated, unbagged	0.007 c	0.39 cd	0.085 c	77.3
5	cut style unpollinated, unbagged	0.024 bc	0.06 d	0.000 c	.
6	abraded stigma pollinated, bagged	0.027 bc	2.40 ab	0.196 bc	34.1
7	abraded stigma pollinated, unbagged	0.009 c	0.39 cd	0.001 c	76.6
8	abraded stigma unpollinated, unbagged	0.077 bc	0.37 d	0.024 c	0.2
9	untreated, open-pollination	0.335 a	1.69 bc	0.564 b	28.0
	Prob (treatment)	<0.001	<0.001	<0.001	n.s.
	Prob (female)	n.s.	n.s.	n.s.	n.s.

6.3.2 Single visit pollination protocol

Seed set was obtained using the method of controlled pollination requiring only a single visit to the *E. globulus* female tree (Table 6.6). This technique involved removing a small part of the style, applying pollen to the tip of the remaining style and isolating it with a small piece of tube, all at anthesis (treatment 3; Table 6.2). This techniques was as, if not more, successful than the traditional three visit method. There was an increase in the number of viable seeds per flower and per capsule with this technique, compared to the

traditional three visit method (treatment 1; Table 6.6), but this was not statistically significant. This slight non-significant increase in seed set with a cut style treatment (treatment 3; Table 6.1) over the traditional three visit treatment (treatment 1; Table 6.1) was also observed in the previous experiment on *E. globulus* (Table 6.3). However, unlike the previous experiment (Table 6.3), the number of seeds produced when applying pollen to the dry stigma (treatment 2; Table 6.6) was reduced significantly ($p < 0.05$) on a seed per capsule basis compared to the single visit cut style method (treatment 3; Table 6.6). The number of flowers setting capsules was also significantly ($p < 0.05$) lower when the dry stigma was pollinated (Table 6.6). When isolating the style with the tube, it was easy to knock pollen off the dry stigma as there is no form of adhesion at anthesis. This may have contributed to the reduction in seed set in pollinating dry stigmas and isolating with tubes (treatment 2; Table 6.6) compared to the same treatment but isolated with bags (treatment 2; Table 6.3).

There was a trend in *E. globulus* at the treatment level for an increase in seeds per capsule to result in a decrease in viable seed weight (Table 6.6). Across all treatments this negative correlation was statistically significant ($p < 0.01$), and the magnitude of the correlation was relatively high ($r = -0.55$). Smaller seeds in *E. globulus* have lower rates of viability, their seedlings have slower early growth rates and a higher levels of seedling abnormalities (Martins-Corder *et al.* 1998). In the present case, the difference in individual seed weight for viable seeds produced from the three visit control method (treatment 1; Table 6. 6) and the single visit technique (treatment 3; Table 6.6) was not statistically significant ($p > 0.05$) suggesting the quality of the seeds produced are comparable.

Table 6.6 Least squares means for different pollination treatments applied to *E. globulus* flowers for the proportion of capsules harvested per flower treated, the number of viable seed per capsule harvested and per flower treated, individual seed weight and percentage of seeds that were inviable. Treatments 4 and 5 (contamination controls) were excluded from the analysis for all traits except capsules per flower. The significance of the effect of pollination treatment and female tree are indicated (n.s. = not significant). Means with the same letter are not significantly different ($p > 0.05$) based on pair-wise contrasts. Flowers were emasculated in all treatments except 9.

No.	Treatment	Capsules per flower	Seeds per capsule	Seeds per flower	Individual seed weight (mg)	% of inviable seeds
1	3 visit control, bag isolated	0.42 ab	27.7 ab	14.1	1.9 b	20.1 b
2	dry style pollinated, tube isolated	0.35 bc	12.7 b	4.7	2.7 a	28.6 ab
3	cut style pollinated, tube isolated	0.55 a	40.0 a	25.7	1.8 b	20.9 b
4	dry style unpollinated, tube isolated	0.0 d	0.0	0.0	.	.
5	cut style unpollinated, tube isolated	0.09 cd	0.0	0.0	.	100
9	Untreated, open pollination	.	10.5 b	.	3.2 a	37.9 a
	Prob (treatment)	<0.01	<0.05	n.s.	<0.001	<0.05
	Prob (female)	n.s.	n.s.	n.s.	n.s.	n.s.

There was no significant influence ($p > 0.05$) of the female tree on the number of seeds set per capsule or per flower, individual seed weight, proportion of inviable seeds or capsule set (Tables 6.3 and 6.6). This is contrary to the finding of Hardner and Potts (1995) who reported that the female trees differ significantly ($p < 0.05$) in these traits. Whether such effects are genetic or environmental remain to be determined.

As the tubes were held to the style by friction at the base, there was some initial concern about contamination should the tubes be knocked off prematurely, as 11.5% of the tubes were missing at debagging (4-5 weeks after pollination).. However, no viable seeds were produced from flowers which were not pollinated but had their styles isolated with tubes

(Table 6.6). There were two capsules collected from the unpollinated cut style treatment, one of which was parthenocarpic whilst the other contained 3 inviable seeds and had developed from a flower which had lost its tube within 2 weeks of anthesis. Self fertilisation results in higher levels of inviable seeds than outcrossing in *E. globulus* (Hardner *et al.* 1998), and would be the most likely origin of these inviable seeds. This case represented less than 2% of the contamination controls, and no contaminated viable seeds were produced in the present study. However, care should be taken to ensure secure tube placement particularly where trees are self-compatible.

Labour and equipment costs are often the largest costs in a controlled crossing programme. By substantially reducing the man-hours required and more effective use of equipment and resources, the production cost per seed can be dramatically reduced. The new method presented here satisfies both requirements and we estimate a reduction in pollination costs of at least 50%. Apart from only needing to visit the flower once, flower wastage is reduced as each flower on a tree can be treated as it becomes ripe and time can be saved as full emasculation is not required (only sufficient to ensure secure placement of the tube). There is also the potential that this method may actually yield slightly more seeds per flower treated, producing a multiplier effect with the time savings achieved.

The results of the present study suggest the single visit pollination method developed for *E. globulus* should be transferable to other eucalypt species with relatively large flowers. The different results obtained for *E. globulus* and *E. nitens* suggests application of the cut style pollination technique to other particularly small flowered species may be problematic. Nevertheless, *E. nitens* has a particularly delicate style and previous success with the small flowered *E. gunnii* (Cauvin 1988) suggests that this pollination technique will be applicable to many smaller flowered species. Additionally, success of a single visit method for small flowered species will require development of a compatible single

flower- or whole umbel- isolation method, which requires no further attention once applied.

The application of the cut style method need not be confined to controlled crossing programmes. It clearly has potential for application in supplementary mass pollination systems where low levels of contamination can be tolerated and flower isolation is unnecessary. When pollen is artificially (supplementary) applied to flowers, they yield more seeds that those naturally open-pollinated (present study, Hardner and Potts 1995, Hardner *et al.* 1988). Our results show that by removing the tip of the pre-receptive style following emasculation at anthesis and applying pollen, the number of seeds per capsule obtained is equivalent to that when pollen is applied to the stigma at peak receptivity. Indeed, this technique should allow the pollination of flowers to be performed over a broader period of development, allowing more operational flexibility. Additionally, as the surface of the cut style may only be receptive for a very short period, compared to the stigma which is receptive for days (Tibbits 1986), the proportion of seeds which would be contaminated by open pollination may be much lower in the cut style treatment and these levels are currently being quantified using molecular means.

Chapter 7

Discussion

This thesis can be divided in to three main themes within the overall aim of improving flowering and seed production in *E. nitens*. Firstly theme 1, *environmental factors* which covered macroenvironmental effects in Chapter 2 and microenvironmental effects in Chapter 3. Secondly theme 2, *silvicultural practices* which covered hormone manipulation and nutrition in Chapters 4 and 5. Finally theme 3, *improving breeding techniques* in Chapter 6.

The macroenvironment, more specifically temperature, has a profound influence on flowering and seed production in *E. nitens* (Chapter 2). An extended period of cold temperatures is widely recognised as a requirement to initiate flowering in *E. nitens* (Eldridge and Griffin 1990, Moncur and Hasan 1994, Swain and Chiappero 1998). Indeed, trees planted in areas which experience frequent frosts and had relatively low minimum temperatures (60 and 640 m sites, Table 2.4) did produce more flowers than sites where frosts were rare and minimum temperatures were relatively high (240 m site, Table 2.4 and the irrigation trial, Table 2.1). However, a cooler climate particularly at higher elevations (440 and 650 m sites, Table 2.4), can be detrimental to reproduction. Trees at higher altitudes flowered later (Table 2.10) and produced seeds which were lighter in weight and had less reliable and slower rates of germination (Figures 2.10 to 2.12). Additionally, there was a trend for fewer seeds per capsule as site elevation increased (Figure 2.9), but this was not significant. Factors which would contribute to poor reproductive performance in cooler conditions are the baseline heat sum requirement for flowering (Moncur *et al.* 1994a) and reduced pollinator activity (Savva *et al.* 1988,

Hingston and Potts 1998). The maternal parent has a major effect on seed production and quality (Table 2.12), whilst there was no evidence of an interaction between maternal environment and germination conditions (Table 2.14). Site quality also appeared to influence reproductive output. Flowering and growth in *E. nitens* responds positively to increased nitrogen nutrition (Chapter 5) and at a cool but low quality site, both growth and flowering were poor (440 m site, Figure 2.5). The most productive site in the study in terms of flower and capsule abundance, seeds per capsule and seed germination was located at only 60m above sea level but would owe much of its success to local topography. This site had the greatest range of temperatures (Table 2.4) and of the lower elevation sites examined, this was the only one to receive frequent frost due to cold air drainage (Beadle *et al.* 1996). Thus this site satisfied both the cold temperature requirement for flower initiation and warmer temperatures for flower development, pollination and seed development.

Subjecting *E. nitens* trees to persistent water stress in the irrigation trial (Chapter 2) enhanced flower and capsule production whilst these traits were substantially reduced by intermittent water stress (Figure 2.2). There is no clear trend in eucalypts in the response to water stress. Water stress can enhance flowering in *E. viminalis* (Moncur 1998) and *E. diversicolor* (Eldridge *et al.* 1993) whilst having the opposite effect in *E. regnans* (Ashton 1975), *E. macrorhyncha* (Ashton and Sandiford 1988) and *E. maculata* (Pook *et al.* 1997). Water availability did not affect reproductive development in the same way as it affected growth in *E. nitens* (Figure 2.2) whereas other treatments to stimulate growth in eucalypts also improve flowering (Cameron and Kube 1983) and suggest the mechanisms are related but discrete.

Flower and capsule production, seed production and seed quality were not affected by maternal drought stress (Tables 2.5 to 2.8) despite the significant effect on growth (Figure

2.2). Flowers opened at the same time and in the same period despite the differences in water availability. This consistency further underlines the importance of accumulated heat sum in flower development proposed by Moncur *et al.* (1994a) as temperatures in the irrigation trial would be reasonably homogeneous. Homogeneity of temperature across the irrigation trial would also provide a more uniform pollinator environment (Savva *et al.* 1988, Hingston and Potts 1998) and this is supported by the number of seeds per capsule. Indeed, neither the number of seed per capsule, seed weight or germination performance were effected by maternal water availability (Tables 2.7 and 2.8) and this suggests a mechanism to protect the development of seeds from maternal water stress (Barnett 1996).

The most important environmental aspect to consider for flowering and seed production in *E. nitens* is temperature. This is also one of the most difficult to control and whilst moving seed production into artificially controlled environments has been suggested (Moncur 1998) this would not be suitable for large scale operations to produce seed for plantation deployment. Ideally sites which satisfy both the chilling requirement for flower induction and warmer temperatures for flower and seed development are the most desirable. Water availability is also an important issue. Conditions which either maintain a high level of drought stress or avoid drought stress altogether support good flowering. However, if high levels of drought stress are alleviated intermittently by watering (or potentially by rainfall), this significantly reduces flowering (Figure 2.2). A hedging approach would be to supply water as needed to avoid drought stress. This approach would also have benefits when treating with paclobutrazol (see review in Chapter 4).

On a microenvironmental scale (Chapter 3), the increased spacing between trees increases the production of flowers and capsules both on a per tree and per hectare basis (Figures 3.4 and 3.6). The maximum number of flowers and capsules produced per hectare was found in the younger (5 years old) of the two trials examined as being between 833 and

500 stem per hectare. The maximum achievable numbers of flowers and capsules per tree in both trials and per hectare in the older trial (13 years old) were not identified as they appear to occur at spacing greater than that available in the trials (i.e. at less than 468 trees per ha). Whilst flower and capsule productivity increase at wider spacings consideration needs to be given to the affect wider spacing has on outcrossing rates and ultimately the quantity and quality of seed production. The viability of seeds harvested from widely spaced *E. grandis* trees was appreciably less than that from trees more closely spaced (van Wyk 1981). Indeed, outcrossing rates can fall sharply as population density decreases (House 1993, Karron *et al.* 1995, Potts and Wiltshire 1997). Further investigation is therefore required to identify what effect spacing has on the quantity and quality of *E. nitens* seeds and to identify the optimum spacing which would balance the sometimes conflicting needs of flower and seed production.

A reduction of growth and apical GA_1 levels in seedlings of *E. nitens* was achieved through the application of the gibberellin biosynthesis inhibitors chlormequat chloride, prohexadione and paclobutrazol but it was not sufficient to induce reproductive development (Chapter 4). In these seedlings, the chilling requirement which induces flowering in adults was met and the level of apical GA_1 (Figure 4.1) was similar to that found in reproductive adult apices of *E. nitens* (Moncur and Hasan 1994). Furthermore, the dose rate of paclobutrazol and method of application used was identical to that which was sufficient to induce reproductive development in seedling of the closely related *E. globulus* (Hasan and Reid 1995). The absence of reproductive development after these treatments suggests an extra level of control over reproductive initiation exists in juveniles of *E. nitens*. To overcome this may requite further chemical or environmental manipulation.

Precocious and abundant flowering in *E. nitens* can be enhanced through treatments to accelerate growth, specifically, fertilisation with nitrogen (Chapter 5) and this effect has also been reported in *E. regnans* (Cameron and Kube 1983). However, improved growth can not explain the full effect which nitrogen has on flowering precocity and abundance (Tables 5.4, 5.8 and 5.10). Nitrogen application enhances flowering in conifers (Bonnet-Masimbert and Webber 1995) and appears to be related to changes in the amino acid and polyamine content of the shoots (Daoudi *et al.* 1994). There may well be such changes in shoots of *E. nitens* but this is yet to be investigated.

Fertilisation of trees with nitrogen and phosphorus also shortened the juvenile vegetative phase of *E. nitens* (Chapter 5). However, the process by which this is achieved by nitrogen and phosphorus was different for each case. Nitrogen accelerated growth rates thus reducing the time in which it takes to reach the size at which the phase change occurs. However, phosphorus did not accelerate growth, instead it appears to directly induce vegetative phase change earlier in the life of the tree.

A further benefit to flower initiation of nitrogen treatment is its effect on improving the efficacy and reliability of paclobutrazol treatment (Figures 5.6 and 5.9). In conifers (Daoudi *et al.* 1994), pears (Raese and Burts 1983) and peaches (George and Nissen 1992) the combination of hormone manipulation with nitrogen fertilisation has substantially increased flowering over that which could be achieved by their application alone. Indeed there may be a synergy between cultural and hormonal treatments in increasing reproductive output (Bonnet-Masimbert and Webber 1995). In *E. nitens*, the effect on flower initiation of the combined treatments appeared to be more than additive (Figure 5.6) though there was no statistical evidence of a synergistic interaction (Table 5.8). However, despite the effectiveness, the dose rates of nitrogen used in Chapter 5 appeared to be suboptimal and treatment with higher rates (> 300 kg.ha^{-1}) combined with

paclobutrazol would be expected to improve flowering further and may show there is indeed a synergy between the treatments.

The need to visit a flower of *E. globulus* to carry out controlled crossing has now been reduced from three visits to one (Chapter 6) and the technique could be expected to be applicable to other eucalypt species with similar sized flowers. Immediately after emasculation at anthesis, the tip of the style can be removed and pollen applied to the fresh cut surface. A short piece of plastic tubing, sealed at one end, is then placed over the pollinated style with a fit to ensure a secure hold at the base (Figure 6.1a-c). Not only does this dramatically reduce the cost and time required to produce controlled crossed seed, it appears to also enhance the number of seed produced for each flower treated (Table 6.6). The style trimming technique will also have application for supplementary mass pollination systems where isolation is not required but the benefit of increased seed yield per flower treated is highly desirable. However, the technique does not appear to be suitable for eucalypt species with small flowers such as *E. nitens*. The style of *E. nitens* is far smaller and more delicate than that of *E. globulus* which makes it prone to damage during style trimming and difficult to effectively place pollen on the cut surface. Little is known about the anatomy of the *E. nitens* style, but when this information becomes available it may be used to modify the current technique to make it more amenable to this species.

Conclusions

The main conclusions to be drawn from this thesis on flowering and seed production in *Eucalyptus nitens* are:

- The flowering season is delayed as site elevation increases at the same or similar latitudes.

- Flower abundance is poorly related to site elevation and is likely to be dependent on combinations of specific microsite factors such as susceptibility to frost and good growing temperatures.

- Seeds harvested from trees growing in cooler sites at higher elevations are lighter in weight and have reduced germination success and rate compared to seeds harvested from trees at lower and warmer sites.

- The number of seeds per capsule can be affected by the number of capsule in an umbel increasing proportionally.

- Flower abundance is affected by maternal water availability.

- Flowering phenology, the number of seeds per capsule, seed weight, germination success and rate is not affected by maternal water availability.

- Variation between individual maternal trees within sites or treatments has a major effect on the number of seeds per capsule, seed weight, germination success and rate.

- Flowering and capsule production increases on a per tree and per hectare basis down to and potentially lower than planting densities of 468 trees per hectare.

- Growth and apical GA_1 levels in seedlings can be reduced by applying either paclobutrazol, chlormequat chloride or prohexadione but flower initiation is not stimulated.

- Nitrogen fertiliser stimulates flower initiation in juvenile and mature trees, mainly through its effect on growth but also acts independently of growth.

- Vegetative phase change occurs earlier on trees planted in phosphorus rich soils.

- The efficacy and reliability of paclobutrazol induced flower initiation in juvenile and mature trees can be improved by the application of nitrogen fertiliser.

Additionally:

- The production of controlled cross seed in *E. globulus* can be achieved with only a single visit to the flower resulting in substantial cost savings in the production of controlled cross seed.

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